THE GEOGRAPHICAL DISTRIBUTION OF SPONTANEOUS ENCEPHALITIS IN RABBITS.

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Since it is always an important matter to secure healthy animals for experimental purposes, considerable attention has been attracted by the discovery that rabbits which are apparently normal are frequently not in reality so, but are actually suffering from a form of encephalitis and nephritis which, in the majority of cases, can only be detected by postmortem histological examination.[1]

Similar lesions, with the parasites which cause them, have also been observed in mice[2] and in cats.[3] The disease may be transmitted to dogs.[4] Spontaneous encephalitic lesions have likewise been reported by Lucke[5] in monkeys, but no accompanying parasites were noted.[6]

[1] The subject is reviewed by Da Fano, C., *Med. Sc.*, 1924, x, 355.

[2] Cowdry, E. V., and Nicholson, F. M., *J. Exp. Med.*, 1924, xl, 51.

[3] Schuster, J., *Klin. Woch.*, 1925, iv, 550.

[4] Cameron, G. C., and Maitland, H. B., *J. Path. and Bact.*, 1924, xxvii, 329. Levaditi, C., Nicolau, S., and Schoen, R., *Ann. Inst. Pasteur*, 1924, xxxviii, 651. The discovery of somewhat similar parasites in dogs suffering from nervous distemper (Kantorowicz, R., and Lewy, F. H., *Arch. wissensch. u. prakt. Thierheilk.*, 1922–23, xlix, 137) and from rabies (Manouelian, Y., and Viala, J., *Ann. Inst. Pasteur*, 1924, xxxviii, 258) is also of interest, particularly in view of a recent paper by Roman and Lapp (Roman, B., and Lapp, C. M., *Buffalo Gen. Hosp. Bull.*, 1925, iii, 40) on the former disease.

[5] Lucke, B., *Arch. Neurol. and Psychiat.*, 1923, x, 212.

[6] A special search for parasites in association with the lesions is not mentioned. The mere existence, however, of apparently spontaneous encephalitic lesions in monkeys is of interest, not only in view of the use of these animals for important experiments, but also as an indication of the possibility of human infection which might conceivably occur in the absence of noticeable symptoms, as it frequently does in rabbits. In order to obtain data on this point, the brains of twenty mon-

In all of these species, as in rabbits, it is exceptional for the diseased animals to exhibit noticeable symptoms. Neither is the brain the only organ concerned. In the rabbit it was early shown that the kidneys are also affected, but it remained for Smith and Florence[7] to demonstrate that the kidneys are primarily involved, and that the parasite is distributed thence *via* the blood stream to all parts of the body.

We are, therefore, confronted by the knowledge that some of the most valuable of our laboratory animals, which belong, indeed, to entirely distinct families, are frequently infested with the same or similar parasites, and, further, that the list of those subject to this insidious disease may not yet be complete.

Not only is the parasite unusually cosmopolitan for a protozoan as to number and variety of the species affected, but also in respect to its wide geographical distribution, because in the 5 years since its discovery it has already been reported from many parts of Europe and the United States. Whether the parasite is spreading from some reservoir in a single species, or whether it has been resident for a considerable period in the several species mentioned above, and possesses but slight invasive ability for other species not yet infested, we do not know. The observation of Oliver[8] that 20 per cent of the rabbits examined at San Francisco in 1921 showed definite encephalitic lesions, whereas 60, examined 4 years earlier (1917), were apparently free from the disease, lends support to the theory that our animal colonies are at present being invaded by the parasite. The failure of investigators

keys, which had been employed for various experimental purposes, were examined. The monkeys, during the experiments, were kept in the same animal house, but usually in different rooms from rabbits and mice known to be heavily infested with the parasites. The examination involved a histological study of twenty blocks of tissue from each of two animals and of five blocks from the remaining eighteen animals. No encephalitic lesions or parasites were found. In order to ascertain whether rats and guinea pigs, under similar conditions, acquire the disease, 114 of the former, and 115 of the latter were examined for parasites with negative results.[2] Encephalitic lesions were occasionally met with, in 0.8 per cent and in 3.8 per cent of the animals respectively, but they did not closely resemble the typical lesions of spontaneous encephalitis of rabbits.

[7] Smith, T., and Florence, L., *J. Exp. Med.*, 1925, xli, 25.

[8] Oliver, J., *J. Infect. Dis.*, 1922, xxx, 91.

to report these lesions, which are so conspicuous and easily detected, before the year 1917[9] may or may not indicate their absence in rabbits, prior to this date.

Acting on the belief that negative observations are of little value, but that an earlier positive description of the disease would be of interest, the large existing literature on the structure of rabbits' brains was searched for reports of the existence of similar lesions or parasites. Special attention was paid to researches which involved the examination of many sections of the cerebral cortex of animals of different ages—as, for example, in studies on the development of cortical lamination—because it is known that the lesions are particularly prone to occur in this region of the brain and that the parasites are usually seen in young animals. But no references to encephalitis were found. A parallel, but less complete, review was made of the literature on the kidneys, similarly without success.

Letters of inquiry were then sent to different centers of neurological research,[10] where rabbits' brains have been favorite objects of study over a period of many years. All answers received were uniformly in the negative. It was thought that it might serve a useful purpose to ascertain whether at the present time the disease occurs in other parts of the world, in addition to the United States and Europe, as a basis for future observations concerning its epidemiology. Other letters were accordingly sent to many laboratories, asking for data regarding the incidence of spontaneous encephalitis, and offering to examine the brains of rabbits being used for experimental purposes, in order to determine its presence or absence. The response was encouraging and showed a widespread desire on the part of investigators, particularly in the tropics, to learn whether this disease was endemic among their animals. Many replies, to the effect that cases of spontaneous encephalitis had not been observed, are to be discounted on

[9] Bull, C. G., *J. Exp. Med.*, 1917, xxv, 557.

[10] (1) Dr. H. H. Donaldson, The Wistar Institute of Anatomy and Biology, Philadelphia, Pa. (2) Dr. C. U. Ariens Kappers, Central Institute for Brain Investigation, Amsterdam, Holland. (3) Dr. C. J. Herrick, Department of Anatomy, University of Chicago, Chicago, Ill. (4) Dr. P. del Rio Hortega, Instituto Cajal, Madrid, Spain. (5) Dr. G. Marinesco, Department of Neurology, University of Bucharest, Bucharest, Roumania.

TABLE I.

No. of rabbits.	Material examined.		Results.
	Source.	Condition.	
10	Rio de Janeiro, Brazil. Dr. R. A. Lambert. Sept., 1924.	Apparently normal.	No encephalitis.
5	Rio de Janeiro, Brazil. Dr. M. M. Metcalf. Oct. 21, 1925.	" "	" "
1	Montevideo, Uraguay. Dr. M. M. Metcalf.	" "	" "
32	Onderstepoort, South Africa. June to Sept., 1924.	13 employed in lamziekte* experiments; 1 severe mange; 18† employed in heartwater‡ experiments.	" "
7	Kabete, Kenya Colony. Dr. James Walker.	Apparently normal.	" "
10§	Bombay, India. Major J. Morison. Aug. 17, 1925.	Dead from some unknown cause.	No encephalitis or nephritis.
9§	Bombay, India. Major J. Morison.	No data.‖	No encephalitis or nephritis.
8§	Calcutta, India. Lt. Col. C. B. Fry. Nov. 3, 1925.	Apparently normal young white rabbits.	No encephalitis or nephritis.
5§	Colombo, Ceylon. Dr. Lucius Nicholls. June 8, 1925.	Apparently normal.‖	No encephalitis or nephritis.

* A South African disease of cattle, caused by a toxin produced by a "bacterium reminiscent but not identical with *B. botulinus*."

† Kidneys, spleen, suprarenals, liver, lungs, and lymph glands examined in addition to brains.

‡ An acute, infectious disease of sheep, goats, and cattle, caused by *Rickettisa ruminantium*.

§ Kidneys examined in addition to brains.

‖ Several of these animals were immature.

TABLE I—*Concluded.*

No. of rabbits.	Material examined.		Results.
	Source.	Condition.	
10§	Colombo, Ceylon. Dr. Lucius Nicholls.	No data.	No encephalitis; slight nephritis in 2.
10§	Nagasaki, Japan. Dr. I. Sibata. July 23, 1925.	Apparently normal.‖	Encephalitis in 3, nephritis in 2, and parasites in 2.
13	Tokyo, Japan. Dr. Michio Inouye. Mar. (?) 1925.	" "	No encephalitis.
13§	Tokyo, Japan. Dr. T. Saito. Aug. 2, 1925.	6 apparently normal; 7 exposed to x-ray.	No encephalitis or nephritis.
15	Peking, China. Dr. W. C. Ma. July, 1924.	Apparently normal.	Encephalitis and parasites in 1.
10§	Victoria, Australia. Dr. W. J. Penfold and Dr. Sawers. Oct. 19, 1925.	5 apparently normal (Australian stock); 5 employed in insulin experiments (English stock).	No encephalitis or nephritis.
11	Melbourne, Australia. Dr. C. H. Kellaway. Oct. 15, 1925.	3 apparently normal; 1 showed diarrhea and paresis; 1 died in epidemic from cause unstated; 6 used for testing localization of streptococci by Rosenau's method.‖	No encephalitis or nephritis.
10§	Adelaide, Australia. Dr. J. B. Cleland. Feb., 1926.	Wild Australian stock apparently normal.	No encephalitis or nephritis.
1§	Adelaide, Australia. Dr. J. B. Cleland. Feb., 1926.	English stock apparently normal.	No encephalitis or nephritis.

the score that the animals were not specially examined with this possibility in mind. Reference may, however, be made to one answer, from the Chief of the Laboratory of Microbiology of the Pasteur Institute at Saigon, French Indo-China. This investigator states that, although he has carefully looked for the disease in a very large number of rabbits, he has failed to find any traces of its occurrence.

The material received for examination, which forms the basis of this report, is summarized in Table I.[11]

In the first column the number of rabbits from which tissues were examined is given. The tissues were fixed in formalin or Zenker's fluid and the sections were colored by Giemsa's stain. In most instances the histological study was limited to the brain, from which transverse sections, cut at five different levels, were examined. But in some (marked "§") the kidneys were also included, sections being taken from two places; while in others (marked "†") the examination was more complete, including, in addition to the brain, the kidneys, spleen, suprarenals, liver, lungs, and lymph glands. These histological studies were often supplemented by the close scrutiny, with a hand lens, of many slices of the brains and kidneys, cut with a razor. In this way pronounced lesions were easily detected, and attention was directed to parts of the brain which otherwise might have escaped more detailed examination.

In the second column the source of the animals is indicated, together with the date when the material was collected, for the reason that Smith and Florence have shown that the disease has a seasonal incidence. There is an increase in the number of cases in the early summer.

The condition of the rabbits examined is noted in the third column. The majority were apparently normal and were selected in a haphazard way from the various animal colonies, but some had been employed for experiments, the character of which is mentioned. These experiments would not, however, obscure any encephalitic or nephritic lesions which might have existed previously. Special attention was directed to animals which were young (marked "‖"), and which, on this account, were more likely to harbor the parasites than the others.

The results of the histological examination are reported in the last column. In arriving at a diagnosis, the distinctive character of the lesions, for which search was being made, as defined particularly by Smith and Florence,[7] and by McCartney,[12] was constantly borne in mind. Other alterations in the brains and kidneys were ignored, unless their existence had a direct bearing upon the subject.

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[11] In addition, twenty-four Swedish rabbits, which had been sent to Dr. Flexner by Dr. Kling, of Stockholm, were thoroughly examined. Two showed both encephalitis and nephritis, one, encephalitis only, and another, only nephritis. No encephalitozoa were observed.

[12] McCartney, J. E., *J. Exp. Med.*, 1924, xxxix, 51.

This survey of 180 rabbits, recruited from different parts of the world, shows that the disease called "spontaneous encephalitis" is not limited geographically to Europe and the United States, but occurs also in China, at Peking, and in Japan, at Nagasaki.

One, out of fifteen Pekingese rabbits, exhibited typical brain lesions, accompanied by parasites. Unfortunately the kidneys were not available for examination. A careful comparison of these lesions and parasites with others observed in New York rabbits revealed the fact that it was impossible to distinguish the one from the other.

The rabbits from Nagasaki were more heavily infested. Encephalitic lesions were observed in three out of ten animals. Pronounced and characteristic nephritis occurred in two of these three, and it is interesting to note that in each rabbit it was limited to one kidney, the other kidney presenting no noticeable lesions of any kind. The parasites were detected in large numbers in association with the encephalitic lesions of one animal and the renal lesions of another. In these animals also, the lesions and parasites seemed identical with others observed in New York rabbits.

The uniformly negative findings in rabbits from the other localities may mean either that the disease is absent, or that an insufficient number of specimens was examined to reveal its presence. The latter interpretation applies particularly to Calcutta, Montevideo, Adelaide and Kabete. Since, however, the twenty-six rabbits from Tokyo and the thirty-two rabbits from Onderstepoort were uniformly negative in respect to the presence of encephalitis, it would at least appear that the percentage of infestation is relatively low at these two places, in the animal colonies from which the specimens were obtained—certainly lower than in the rabbits of The Rockefeller Institute at New York, where it amounts to as much as 76 per cent of certain stocks.[12]

SUMMARY.

The observations reported in this paper seem to justify the hope that the obstacle afforded to experimentation by the frequent presence of spontaneous encephalitis and nephritis in American and European rabbits does not exist to the same degree in rabbits from certain other localities. The observations are, furthermore, despite their obvious

limitations, in conformity with the theory that the disease is not one common to domestic rabbits everywhere, but rather that it occurs in special localities from which, given favorable conditions, it may spread. Thus far it has not been reported in tropical climates or south of the equator.

ORGAN WEIGHTS OF NORMAL RABBITS.

Second Paper.

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In a previous paper (1), we reported results of a series of organ weight determinations on a group of 350 normal rabbits that were killed and examined between January 1, 1922, and July 1, 1924. The observations on which this report was based were continued for another year with a view to obtaining data covering a longer period of time and a second set of results which might be compared and combined with the first, thus affording material for a more comprehensive and more exact survey of conditions that obtain in normal rabbits.

The object of the present paper is to record the results of the second series of weight determinations and the results for the entire group of 645 rabbits.

Methods and Material.

The results to be reported are based on a study of two series of male rabbits representing carefully selected normal stocks. The first series (Series I) contained 350 animals; these were killed and examined between January 1, 1922, and July 1, 1924. The methods employed in conducting the investigation are described in detail elsewhere (1). The second series (Series II) was composed of 295 rabbits comparable in all respects to those of Series I and the investigation was carried out in the same manner. These animals were killed and examined in small groups, at intervals of 2 to 4 weeks, between July 1, 1924, and July 1, 1925. The total number of animals is, therefore, 645 and the time covered by the observations is 3½ years.

RESULTS.

The results are recorded in the form of a tabulated summary (Table I) which gives values for actual weights and for weights per kilo of

TABLE I.

Values for Actual and Relative Weights for Rabbits of Series I and Series II and for the Total of 645 Rabbits.

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Gross body weight.	I	350	2265	2225	1400	3500	352.8	237.9	15.57
	II	295	2257	2225	1600	3200	306.3	206.6	13.56
		645	2262	2225	1400	3500	336.4	226.9	14.85
Gastrointestinal mass.	I	350	416.4	410	115	710	80.9	54.5	19.42
Actual.	II	295	424.4	423	195	740	79.3	53.5	18.68
		645	419.9	415	115	740	80.8	54.5	19.24
Relative.	I	350	229.7	226	70.4	426	52.1	35.1	22.68
	II	295	235.2	230	118.0	452	49.9	33.7	21.21
		645	232.2	229	70.4	452	51.2	34.5	22.03
Net body weight.	I	350	1848.5	1800	1150	3010	323.3	218.0	17.49
	II	295	1833.1	1800	1210	2660	273.0	184.1	14.89
		645	1841.0	1800	1150	3010	301.3	203.2	16.36
Heart.	I	348	5.26	5.12	3.2	10.18	0.96	0.64	18.23
Actual.	II	295	5.31	5.22	3.3	9.02	0.93	0.627	17.51
		643	5.28	5.15	3.2	10.18	0.938	0.63	17.83
Relative.	I	348	2.85	2.81	1.95	4.42	0.35	0.24	12.32
	II	295	2.90	2.88	2.04	4.36	0.339	0.229	11.68
		643	2.87	2.82	1.95	4.42	0.344	0.232	11.98

Liver.	I	350	84.3	80.0	50.0	150.0	19.7	13.3	23.39
Actual.	II	295	87.5	85.0	47.0	185.0	19.3	13.0	22.04
		645	85.8	85.0	47.0	185.0	19.6	13.2	22.84
Relative.	I	350	46.5	44.9	23.2	97.4	11.8	8.0	25.48
	II	295	48.5	46.7	25.8	117.0	12.1	8.2	24.96
		645	47.4	45.8	23.2	117.0	11.9	8.0	25.10
Kidneys.	I	348	12.70	12.50	7.15	22.73	2.14	1.44	16.85
Actual.	II	294	13.22	13.05	8.33	20.00	2.06	1.39	15.55
		642	12.94	12.60	7.15	22.73	2.14	1.44	16.53
Relative.	I	348	6.97	6.84	3.45	17.28	1.26	0.85	18.08
	II	294	7.32	7.23	4.29	12.80	1.21	0.82	16.55
		642	7.13	6.98	3.45	17.28	1.25	0.84	17.53
Spleen.	I	347	0.990	0.900	0.08	3.20	0.436	0.294	44.04
Actual.	II	295	1.065	0.985	0.16	5.05	0.549	0.370	51.54
		642	1.023	0.940	0.08	5.05	0.512	0.345	50.04
Relative.	I	347	0.531	0.490	0.035	1.714	0.214	0.144	40.30
	II	295	0.587	0.528	0.107	2.930	0.326	0.220	55.53
		642	0.564	0.508	0.035	2.930	0.284	0.192	50.35
Thymus.	I	349	2.210	2.13	0.45	5.90	0.848	0.572	38.37
Actual.	II	295	2.360	2.25	0.37	6.30	0.842	0.568	35.67
		644	2.301	2.24	0.37	6.30	0.845	0.570	36.72
Relative.	I	349	1.230	1.147	0.334	2.720	0.451	0.304	36.64
	II	295	1.304	1.269	0.248	3.315	0.464	0.313	35.58
		644	1.264	1.215	0.248	3.315	0.463	0.312	36.62

TABLE I—*Continued.*

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Testicles.	I	289	4.86	4.85	0.81	9.12	1.630	1.100	33.54
Actual.	II	294	4.50	4.48	0.92	9.01	1.415	0.954	31.44
		583	4.67	4.70	0.81	9.12	1.520	1.030	32.54
Relative.	I	289	2.61	2.62	0.470	4.93	0.729	0.492	27.93
	II	294	2.45	2.42	0.562	4.22	0.650	0.440	26.53
		583	2.53	2.53	0.470	4.93	0.708	0.478	27.98
Brain.	I	150	9.31	9.24	7.42	12.03	0.780	0.526	8.37
Actual.	II	295	9.11	9.15	5.80	11.33	0.687	0.463	7.54
		445	9.17	9.20	5.80	12.03	0.754	0.508	8.22
Relative.	I	150	5.11	5.06	3.33	8.16	0.870	0.580	16.92
	II	295	5.06	5.01	3.45	7.08	0.678	0.457	13.39
		445	5.08	5.02	3.33	8.16	0.743	0.501	14.62
Thyroid.	I	349	0.23048	0.185	0.085	1.570	0.16209	0.10933	70.33
Actual.	II	295	0.23560	0.210	0.085	1.730	0.13260	0.08944	56.28
		644	0.23280	0.200	0.085	1.730	0.14750	0.09949	63.35
Relative.	I	349	0.12324	0.0975	0.0504	0.730	0.07570	0.05106	61.47
	II	295	0.13030	0.1149	0.0481	1.230	0.08119	0.05476	62.31
		644	0.12650	0.1095	0.0481	1.230	0.07767	0.05239	61.39
Parathyroids.	I	349	0.01256	0.0120	0.0020	0.035	0.00486	0.00328	38.69
Actual.	II	292	0.01320	0.0120	0.0030	0.035	0.00551	0.00372	41.74
		641	0.01286	0.0120	0.0020	0.035	0.00508	0.00343	39.50

Relative.	I	349	0.00692	0.00631	0.00109	0.02175	0.00278	0.00187	40.13
	II	292	0.00730	0.00667	0.00178	0.01850	0.00289	0.00195	39.60
		641	0.00709	0.00642	0.00109	0.02175	0.00283	0.00191	39.91
Hypophysis.	I	348	0.0280	0.028	0.015	0.044	0.00517	0.00349	18.46
Actual.	II	294	0.0283	0.028	0.015	0.050	0.00553	0.00373	19.55
		642	0.0281	0.028	0.015	0.050	0.00526	0.00355	18.72
Relative.	I	348	0.01540	0.01530	0.00728	0.0294	0.00310	0.00209	20.27
	II	294	0.01566	0.01535	0.00780	0.0352	0.00322	0.00217	20.56
		642	0.01550	0.01530	0.00728	0.0352	0.00316	0.00213	20.38
Suprarenals.	I	349	0.3858	0.358	0.115	1.050	0.15460	0.10428	40.07
Actual.	II	295	0.3802	0.350	0.138	1.050	0.16460	0.11102	43.29
		644	0.3833	0.350	0.115	1.050	0.15950	0.10760	41.61
Relative.	I	349	0.2088	0.1967	0.0804	0.530	0.07503	0.05061	35.93
	II	295	0.2078	0.1850	0.0803	0.572	0.08460	0.05706	40.71
		644	0.2082	0.1920	0.0803	0.572	0.07950	0.05360	38.18
Pineal.	I	348	0.01609	0.014	0.005	0.030	0.00429	0.00289	26.66
Actual.	II	292	0.01540	0.015	0.005	0.040	0.00501	0.00338	32.53
		640	0.01577	0.015	0.005	0.040	0.00465	0.00314	29.48
Relative.	I	348	0.00885	0.00868	0.00273	0.01873	0.00245	0.00165	27.68
	II	292	0.00853	0.00836	0.00228	0.02470	0.00290	0.00196	33.99
		640	0.00871	0.00853	0.00228	0.02470	0.00266	0.00179	30.53
Popliteal lymph nodes.	I	242	0.25539	0.245	0.085	0.720	0.08241	0.05559	32.27
Actual.	II	294	0.25612	0.245	0.090	0.570	0.07690	0.05187	30.02
		536	0.25570	0.245	0.085	0.720	0.07940	0.05360	31.05

TABLE I—*Concluded.*

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Popliteal lymph nodes.	I	242	0.13573	0.1361	0.0525	0.3820	0.04873	0.03287	35.90
Relative.	II	294	0.14250	0.1388	0.0500	0.3335	0.04415	0.02978	30.98
		536	0.14066	0.1380	0.0500	0.3820	0.04620	0.03116	32.84
Axillary lymph nodes.	I	242	0.17585	0.175	0.040	0.400	0.06141	0.04142	34.92
Actual.	II	292	0.16320	0.157	0.070	0.485	0.0562	0.03790	34.43
		534	0.16970	0.165	0.040	0.485	0.05909	0.03986	34.82
Relative.	I	242	0.09722	0.0910	0.01865	0.2425	0.03779	0.02549	38.87
	II	292	0.09005	0.0856	0.03410	0.2400	0.03170	0.02138	35.20
		534	0.09368	0.0875	0.01865	0.2425	0.03480	0.02347	37.14
Deep cervical lymph nodes.	I	124	0.15579	0.140	0.035	0.405	0.0732	0.04938	46.98
Actual.	II	289	0.15640	0.130	0.050	0.660	0.0787	0.05308	50.31
		413	0.15623	0.135	0.035	0.660	0.0765	0.05160	48.96
Relative.	I	124	0.08207	0.0724	0.02052	0.211	0.03712	0.02504	45.23
	II	289	0.08580	0.0738	0.03070	0.295	0.04140	0.02790	48.25
		413	0.08467	0.0739	0.02052	0.295	0.04022	0.02713	47.50
Mesenteric lymph nodes.	I	133	3.488	3.32	1.34	9.19	1.310	0.880	37.53
Actual.	II	293	3.460	3.34	1.07	11.47	1.133	0.764	32.75
		426	3.460	3.34	1.07	11.47	1.190	0.803	34.39
Relative.	I	133	1.828	1.763	0.72	3.71	0.642	0.433	35.12
	II	293	1.915	1.830	0.67	6.91	0.642	0.433	33.52
		426	1.885	1.825	0.67	6.91	0.642	0.433	34.05

net body weight (relative weight) as defined in the first paper (1). In each case, the results for Series I and II and for the total are recorded separately in the order mentioned. It will be noted that in the case of the gross body weight, the kidneys (actual), testicles (relative), suprarenals (actual), and axillary lymph nodes (actual), the figures given for Series I differ in a few respects from those given in the original paper. The changes represent corrections of slight errors in the original figures.

DISCUSSION AND CONCLUSIONS.

The results recorded in Table I require very little comment. It is at once apparent that there is a remarkably close agreement between corresponding values for Series I and Series II. In many instances, the agreement is almost absolute despite the occurrence of wide variations in the weights of individual organs. For example, the differences recorded for mean weights of Series I and II lie between 0.296 and 0.008 times the probable error of the difference; the values mentioned are those for the relative weights of the kidneys and the actual weights of the deep cervical lymph nodes, respectively. The closeness of the agreement thus shown is highly significant in view of the magnitude of the probable errors.

It is evident that, while any one of the three groups of figures would give an acceptable measure of mean weights and of the variation in weight of the organs concerned, neither the standard deviation, the probable error, nor the coefficient of variation gives more than a vague idea of the magnitude of the difference that may be found when mean weights are compared. In all cases, this difference proves to be much smaller than would be expected. This fact in itself suggests that the variations in weight are not due entirely to random sampling but a discussion of the reasons for the discrepancy between actual findings and theoretical expectations must be deferred until the material has been analyzed with reference to the occurrence of variations. Meantime, we may emphasize the fact that it is possible to obtain a remarkably close agreement of all values for organ weight, irrespective of whether the variations in weight are large or small, provided a sufficient number of observations is made and the observations are so distributed in time as to permit equalization of the

effects of factors that cause these variations. Similar conditions obtain for smaller groups of animals provided the conditions under which the determinations are made are constant or closely comparable, but as the numbers are decreased and the conditions varied the agreement becomes less and less until the differences found may reach those indicated by the probable error or standard deviation. Still, the difference between weights for groups of even ten animals rarely exceeds these limits which are based on the weights of individual organs and not on group averages.

Finally, in submitting these values for normal stock rabbits, it seems advisable to caution against the acceptance of such values as standard measures of organ weight applicable under all circumstances. These investigations have shown that for most organs it is not possible to obtain values that represent anything more than rough approximations to conditions that may be found at any particular time or under any particular set of natural circumstances. The values for mean weight, as recorded above, are to be regarded merely as focal points about which weights in general may fluctuate with upper and lower limits of probable variation of individual organs and of mean weights fixed by the probable error or the standard deviation. A mean weight may fall anywhere within the limits thus fixed or in the case of the weight of an individual organ, it may exceed these limits (*cf.* maximum and minimum values) and still be within the range of normal. In like manner, the median and the mode indicate approximate midpoints and points of greatest weight frequency, while the coefficients of variation serve as a general index of the degree of uniformity that may be expected for different classes of organs and of variation in the weights of individual organs of any given class. Naturally, the smaller the coefficient the closer the agreement that may be expected under a given set of circumstances as the results are less apt to be disturbed by irregularities in the weights of individual organs or the occurrence of organs whose weights are exceptionally large or small.

The limitations that must be imposed upon the acceptance of any value as a standard of normality will become apparent when we consider the values obtained for the various small groups comprising this series and factors that affect these values. For the present, it is

sufficient to say that this study of organ weights has shown that we are not justified in attempting to fix rigid standards of normality for organ weight. All values of which we have any knowledge are subject to wide variation and what is strictly normal under one set of conditions may be decidedly abnormal under another; existing conditions determine what the normal value should be in a given case. The most that we can do is to define certain broad limits within which such values lie. This we have attempted to do in Table I.

SUMMARY.

The results of a second series of organ weight determinations on normal rabbits are reported and the values obtained are compared with those for the first series. Figures are also given which represent the results obtained by combining the two series of animals or from weight determinations made on 645 apparently normal rabbits selected from stocks used for various experimental purposes.

BIBLIOGRAPHY.

1. Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1925, xlii, 69.

STUDIES ON REGENERATION OF BACTERIOPHAGE.

II. The Influence of Oxygen upon the Behavior of Bacillus coli towards Lytic Principle.

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In studies on bacteriophage the rôle of oxygen has not been thoroughly investigated. d'Hérelle has come to the conclusion that the presence or absence of oxygen has no effect upon the course of this phenomenon (1). Later Gildemeister and Herzberg found that lysis does not depend upon the pressure of oxygen in a culture containing a *low* dilution of bacteriophage (2). On the other hand in a previous communication by the author of this paper it was shown that a definite restriction in supply of oxygen is essential for regeneration of certain *extreme* dilutions of lytic principle (3). In view of these contradictory findings the latter observation suggested that oxygen plays a definite but masked rôle, which becomes evident only on creating unfavorable conditions, as may be the case when an extreme dilution is used.

The object of this paper was to extend the author's above mentioned observations by determining the mechanism by which oxygen interferes with the bacteriophage phenomenon. For this purpose studies were made of the following:

I. The influence of oxygen upon the behavior of *B. coli* towards lytic principle.

II. The direct influence of oxygen upon diluted and undiluted lytic principle.

III. The relation of possible changes in the rate of growth of *B. coli* under restricted supply of oxygen to regeneration of highly diluted lytic principle.

IV. The influence of anaerobiosis and aerobiosis upon absorption of lytic principle by a strain of *B. coli* susceptible to bacteriophage.

I.

To explain the author's findings on the relation of oxygen to regeneration of highly diluted lytic principle it was assumed that restriction in supply of oxygen may be able to create certain changes in the bacterial cell itself—probably by modifying in some way the respiratory function—which changes are extremely favorable for regeneration of lytic principle.

To prove this assumption experiments were undertaken in which it was attempted to induce changes in the behavior of *B. coli* towards lytic principle by preliminary cultivation in the presence or absence of oxygen and then to determine the characteristic features of such changes.

In view of the close correlation existing between the respiratory function of the living cell and hydrogen ion concentration, anaerobic and aerobic cultures of *B. coli* were made in media of various pH.

Methods.

The work described in this paper was carried out on a strain of *B. coli* which was daily transplanted on agar slants. In this way its high susceptibility to the anti-*coli* lytic principle employed in this laboratory was constantly preserved. It is necessary to mention here that this strain was always self-agglutinable in broth cultures, but the sediment was easily broken up on slight shaking.

To obtain *anaerobic* cultures the inoculum was seeded into broth previously autoclaved or boiled for 10 to 15 minutes and the cultures covered with a layer of sterile vaseline.

Aerobic cultures were grown in flasks or large tubes in quantities of fluid which allowed a ratio of surface area exposed to oxygen to total volume of fluid of not less than 3.5.

The *lytic principle* used in these experiments was the same that was used in work previously reported (3).

For the determination of possible changes in *behavior* of the strain towards lytic principle, observations on susceptibility to bacteriophage as well as on the bacteriophage-regenerating property were taken as criteria.

Changes in susceptibility to bacteriophage were determined by the degree of lysis in broth culture containing a low dilution of lytic principle,

$$\left(\frac{\text{Surface area}}{\text{Total volume}} \text{ ratio} = 3.5\right)$$

and by the clearness of the eaten up area produced by a drop of lytic principle deposited on a dry agar slant previously seeded with the microorganism to be tested.

Changes in the bacteriophage-regenerating power of the microorganism were determined in a more or less quantitative manner by finding the highest dilution of lytic principle which a given amount of bacterial cells is capable of regenerating to its full strength.

EXPERIMENTAL.

Anaerobic and aerobic cultures of *B. coli* each grown in meat infusion broth of pH 6.0, 7.0, 7.6, 8.4, were incubated for 24 hours and then kept at room temperature for 2 days. The behavior of these eight cultures towards lytic principle was tested.

TABLE I.

Effect of Cultivation of B. coli in the Presence and Absence of Oxygen upon Its Susceptibility towards the Lytic Principle as Demonstrated by the Degree of Lysis in Broth Cultures. $\left(\frac{\text{Surface area}}{\text{Total volume}} \text{ ratio} = 3.5\right)$

Source of inocula.	Aerobic culture. pH 6.0	Anaerobic culture. pH 6.0	Aerobic culture. pH 7.0	Anaerobic culture. pH 7.0	Aerobic culture. pH 7.6	Anaerobic culture. pH 7.6	Aerobic culture, pH 8.4	Anaerobic culture. pH 8.4
Degree of lysis in 1 cc. broth containing 0.02 cc. of 10^{-2} of bacteriophage. $\left(\frac{\text{Surface area}}{\text{Total volume}} \text{ ratio} = 3.5\right)$	4+	4+	4+	1+	1+	4+	4+	4+

1+ indicates slight lysis; 4+ indicates complete lysis.

The Effect of Cultivation of B. coli under Aerobic and Anaerobic Conditions upon Its Susceptibility to Lysis.

Emulsions of equal turbidity were prepared from anaerobic and aerobic cultures of pH 6.0, 7.0, 7.6, 8.4, and 0.05 cc. of each was inoculated into 1 cc. of broth of pH 7.0 containing 00.2 cc. of 10^{-2} dilution of lytic principle

$$\left(\frac{\text{Surface area}}{\text{Total volume}} \text{ ratio} = 3.5\right).$$

The reading of the degree of lysis obtained in 24 hours of incubation is recorded in Table I.

Identical results were obtained by testing the resistance of aerobic and anaerobic cultures of various initial pH by depositing a drop of

lytic principle on the surface of agar slant subcultures. The results are shown in Table II.

As is seen from Tables I and II, the resistance of *B. coli* to lytic principle is a property which can be induced by cultivating this microorganism under anaerobic conditions at initial pH 7.0 or under aerobic conditions at initial pH 7.6. At distinctly acid and alkaline reactions the absence or presence of oxygen does not interfere with the behavior of *B. coli* towards bacteriophage.

Effect of Cultivation of B. coli under Aerobic and Anaerobic Conditions upon Its Bacteriophage-Regenerating Property.

The anaerobic and aerobic cultures of *B. coli* of various initial pH prepared in the manner described above were each inoculated into a series of eight tubes containing 1 cc. of meat infusion broth of pH 7.0 $\left(\frac{\text{Surface area}}{\text{Total volume}} \text{ ratio} = 3.5\right)$ Dilutions of lytic principle from 0.1 cc. $\times 10^{-1}$ up to 0.1 cc. $\times 10^{-8}$ were made in each series of eight tubes and all the tubes incubated at 37°. In 24 hours the degree of lysis in each tube was noted. All the tubes were then centrifuged, their supernatant fluid heated at 58° for ½ hour, and the lytic exponent of all these samples determined by serial dilution in broth. Table III represents the results obtained.

As is seen from this table the appearance of resistance to bacteriophage under anaerobic conditions at pH 7.0 and under aerobic conditions at pH 7.6 is coincident with a considerable loss in the regenerating power of these strains.

Stability of Resistance Induced in the Presence and Absence of Oxygen.

The object of experiments described below was to find out whether the changes in susceptibility imparted by cultivation of a susceptible strain of *B. coli* in the presence and absence of oxygen at various initial pH can be transmitted to new generations of these strains provided the original conditions as imposed on the parent strains be continued. For this purpose every 48 hours all the aerobic and anaerobic cultures were subcultured under conditions similar to those of the parent cultures for a period of 40 days and the resistance to bacteriophage of every series of subcultures tested in the manner described above. In all these experiments exactly identical results were obtained as with the parent cultures.

TABLE II.

Effect of Cultivation of B. coli in the Presence and Absence of Oxygen upon Its Susceptibility towards the Lytic Principle as Demonstrated by the Appearance of Eaten Up Areas.

Type of cultures used for inoculation of agar slants.	Aerobic culture. pH 6.0	Anaerobic culture. pH 6.0	Aerobic culture. pH 7.0	Anaerobic culture. pH 7.0	Aerobic culture. pH 7.6	Anaerobic culture. pH 7.6	Aerobic culture. pH 8.4	Anaerobic culture. pH 8.4
Appearance of eaten up area on agar slants.	Clear area. No resistants.	Clear area. No resistants.	Clear area. No resistants.	Indistinct area. Numerous resistants.	Indistinct area. Numerous resistants.	Clear area. No resistants.	Clear area. No resistants.	Clear area. No resistants.

TABLE III.

Effect of Cultivation of B. coli under Aerobic and Anaerobic Conditions at Various Initial pH upon Its Bacteriophage-Regenerating Power.

Dilution of bacteriophage in 1 cc. of broth. ($\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 3.5)	10^{-1}		10^{-2}		10^{-3}		10^{-4}		10^{-5}		10^{-6}		10^{-7}		10^{-8}	
Source of inocula.	Degree of lysis.	E_L of regenerated bacteriophage.	Degree of lysis.	E_L of regenerated bacteriophage.	Degree of lysis.	E_L of regenerated bacteriophage.	Degree of lysis.	E_L of regenerated bacteriophage.	Degree of lysis.	E_L of regenerated bacteriophage.	Degree of lysis.	E_L of regenerated bacteriophage.	Degree of lysis.	E_L of regenerated bacteriophage.	Degree of lysis.	E_L of regenerated bacteriophage.
Aerobic culture pH 6.0.	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8
Anaerobic culture pH 6.0.	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8
Aerobic culture pH 7.0.	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8
Anaerobic culture pH 7.0.	1+	8	1+	8	0	8	0	8	0	0	0	0	0	0	0	0
Aerobic culture pH 7.6.	1+	8	1+	8	0	8	0	8	0	0	0	0	0	0	0	0
Anaerobic culture pH 7.6.	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8
Aerobic culture pH 8.4.	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8
Anaerobic culture pH 8.4.	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8	4+	8

4+ indicates complete lysis; 1+ indicates slight lysis; 0 indicates no lysis.

Inability of Lytic Principle to Overcome the Resistance Created under Aerobic and Anaerobic Conditions.

To establish whether the lytic principle is able to adapt itself to the resistant strains so as to gain the property of producing lysis, the following experiment was undertaken.

Tubes of broth containing 10^{-2} dilution of lytic principle were inoculated respectively with aerobic culture of pH 7.6 and with anaerobic culture of pH 7.0. In 24 hours of incubation the cultures were centrifuged, and their supernatant fluid heated to 58° for ½ hour. These samples were added to broth in dilution 10^{-2} and then the dilutions inoculated with 24 hour old subcultures of corresponding strains of *B. coli*. This process of passage of lytic principle always through the same type of resistant strain was repeated ten times. After each passage the ability of the newly regenerated lytic principle to produce lysis of aerobic and anaerobic resistant cultures was tested. The results are shown in Table IV.

TABLE IV.

Inability of Lytic Principle to Overcome the Resistance Created under Aerobic Conditions at pH 7.6 and Anaerobic Conditions at pH 7.0.

Broth 1 cc. + 0.1 cc. of 10^{-2} dilution of bacteriophage regenerated by aerobic culture pH 7.6 (passages 1–10).	
Degree of lysis of aerobic culture pH 7.6.	Degree of lysis of susceptible strain.
0	4+
Broth 1 cc. + 0.1 cc. of 10^{-2} dilution of bacteriophage regenerated by anaerobic culture pH 7.0 (passages 1–10).	
Degree of lysis of anaerobic culture pH 7.0.	Degree of lysis of susceptible strain.
0	4+

These results show the inability of the lytic principle to overcome the resistance of the aerobic and anaerobic resistant cultures.

Identity of Action of Lytic Principles Regenerated by Resistant Aerobic and Anaerobic Cultures.

It was seen from the experiments on the bacteriophage-regenerating power of resistant aerobic and anaerobic cultures that this power is retained to a certain extent (Table III). It was therefore considered of interest to investigate whether lytic principles identical in action are regenerated by both aerobic and anaerobic types of resistant

strains. For this purpose the lytic principles mentioned in the previous experiment which were obtained after ten passages through aerobic cultures of pH 7.6 and anaerobic cultures of pH 7.0 were tested each against *B. coli* aerobic and anaerobic cultures of various initial pH. For control the lytic principle employed in this laboratory was similarly tested. The results are recorded in Table V.

This experiment shows clearly that the lytic principles regenerated at the expense of resistant aerobic and anaerobic cultures are identical in action with each other as well as with the stock lytic principle.

The above described experiments show that oxygen plays a definite

TABLE V.

Identity of Action of Lytic Principles Regenerated by Resistant Aerobic and Anaerobic Cultures.

	Degree of lysis of aerobe pH 6.0.	Degree of lysis of anaerobe pH 6.0.	Degree of lysis of aerobe pH 7.0.	Degree of lysis of anaerobe pH 7.0.	Degree of lysis of aerobe pH 7.6.	Degree of lysis of anaerobe pH 7.6.	Degree of lysis of aerobe pH 8.4.	Degree of lysis of anaerobe pH 8.4.
Broth 1 cc.+0.1 cc. 10^{-3} dilution of bacteriophage regenerated by anaerobe, pH 7.0 (10th passage).	4+	4+	4+	0	0	4+	4+	4+
Broth 1 cc.+0.1 cc. 10^{-3} dilution of bacteriophage regenerated by aerobe, pH. 7.6 (10th passage).	4+	4+	4+	0	0	4+	4+	4+
Broth 1 cc.+0.1 cc. 10^{-3} dilution of stock bacteriophage.	4+	4+	4+	0	0	4+	4+	4+

rôle in the bacteriophage phenomenon by influencing the behavior of *B. coli* towards this principle.

Since the object of this paper was to establish the rôle of oxygen in the bacteriophage phenomenon it remained to determine whether any other rôle can be attributed to this factor

II.

The direct influence of air and oxygen was studied by aerating for several hours or bubbling pure oxygen through diluted and undiluted sterile lytic principle previously adjusted to various hydrogen ion concentrations. The lytic potency of aerated and oxygenated samples

was then estimated by making serial dilutions in broth. In no case was any inactivation or weakening of lytic principle ever observed under the influence of air or oxygen.

III.

In the author's previous paper on the regeneration of bacteriophage under partial anaerobiosis (3) it was reported that when the original inoculum is sufficiently large no difference between the rates of bacterial growth under free and restricted supplies of air can be discovered. Therefore, it was assumed in this paper that partial anaerobiosis does not favor regeneration of highly diluted lytic principle by modifying the rate of bacterial growth. But further investigation of this point was desirable since there is an appreciable error in counting viable microorganisms and, especially when, as in this case, a large number has to be counted.

For the final study of this point our knowledge on the relation of the size of the original inoculum to the rate of growth (4) was made use of as in the following experiment.

Samples of 5 cc. of broth containing highly diluted lytic principle (10^{-9}) were seeded in duplicate with various numbers of *B. coli* ranging from 10 up to 100 million per cc. One series of samples of broth was then poured into tubes of 100 by 8 mm. in size and another into tubes of the size usually employed for bacteriological work.

Since the size of the original inoculum varied considerably in this experiment all possible rates of growth differing in length of various phases and number of bacteria at each phase were obtained in both types of containers as was shown by bacterial counts. However in 24 hours of incubation no lysis occurred under free supply of air irrespective of the rate of growth, but lysis invariably occurred under restricted supply of air. It is evident, therefore, that the rate of bacterial growth has no relation whatsoever to regeneration of certain extreme dilutions of bacteriophage under restricted supply of air.

IV.

To establish whether the supply of air has any influence upon absorption of lytic principle by susceptible bacterial cells—the following experiment was carried out.

24 hour old agar slant cultures of *B. coli* strain susceptible to bacteriophage were washed off with salt solution. The emulsion was centrifuged and resuspended in 10^{-5} broth dilution of lytic principle in order to obtain an emulsion corresponding in density to that of No. 5 tube of McFarland's nephelometer. This emulsion was then distributed in 1 cc. quantities into a series of tubes. The fluid of one half of these tubes was covered with a layer of sterile vaseline. All the tubes were kept at $-4°$ to $-8°$ for various intervals of time from 2 up to 24 hours. Afterwards the tubes were centrifuged, supernatant fluid collected and heated at 58° for ½ hour, and the lytic potency of each sample determined by serial dilutions in broth.

In no case was there observed any difference in the rate of absorption of lytic principle under anaerobic and aerobic conditions.

SUMMARY AND CONCLUSIONS.

The investigations of the influence of oxygen on the bacteriophage phenomenon recorded in this paper show that this factor plays an important rôle which is due exclusively to its ability to induce certain changes in the behavior of bacterial cells towards lytic principle. At certain H ion concentrations both aerobic and anaerobic cultures can be made resistant in the absence of lytic principle. The resistance thus acquired is of a stable nature under suitable conditions. If the lytic principle is added to aerobic or anaerobic types of resistant strains the cultures are able to regenerate it to a certain extent. However they do not undergo any visible lysis themselves even under the action of lytic principles which were passed through several generations of these types. The principles regenerated by both types of resistant cultures are identical in action with each other as well as with the stock lytic principle.

These experiments suggest a new method of investigation into the hitherto unexplained nature of resistance of bacteria towards bacteriophage.

BIBLIOGRAPHY.

1. d'Hérelle, F., The bacteriophage, English translation by Smith, G. H., Baltimore, 1922, 53.
2. Gildemeister, E., and Herzberg, K., Ueber das d'Herellesche Phänomen. III. Mitteilung, *Centr. Bakt., I. Abt., Orig.*, 1923–24, xci, 12.
3. Shwartzman, G., Studies on regeneration of bacteriophage. I. The influence of partial anaerobiosis upon regeneration of a highly diluted lytic principle, *J. Exp. Med.*, 1925, xlii, 507.
4. Penfold, W. J., On the nature of baterial lag, *J. Hyg.* 1914, xiv, 214.

STUDIES ON UROBILIN PHYSIOLOGY AND PATHOLOGY.

VI. The Relation of Biliary Infections to the Genesis and Excretion of Urobilin.

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In previous papers from this laboratory it has been shown that the normal presence of urobilin in the body is dependent upon the entrance of bile pigment into the intestine.[1–5] Complete bile deprivation, by intubation of the common duct with collection of the total bile in such a way as to maintain sterility, is followed by the total disappearance of urobilin from the excreta, not only in the normal animal but even after the liver is damaged, the bile ducts occluded, or blood destroyed.[4,5] These findings are not in accord with the experiments of many previous workers,[6,7] but these latter employed the open bile fistula methods and dealt necessarily with infected biliary tracts. We now propose to discuss the influence of infection in the light of our own further work, in which we have deliberately infected intubated and previously sterile duct systems with urobilin-producing bacteria. It is of both practical and theoretical importance to determine whether during biliary obstruction or upon liver damage the urobilin formed in an infected biliary tract can be absorbed therefrom and lead to the appearance of the pigment in the urine.

It has long been known that urobilin is formed in the intestine by a

[1] Elman, R., and McMaster, P. D., *J. Exp. Med.*, 1925, xli, 503.
[2] McMaster, P.D., and Elman, R., *J. Exp. Med.*, 1925, xli, 513.
[3] McMaster, P.D., and Elman, R., *J. Exp. Med.*, 1925, xli, 719.
[4] Elman, R., and McMaster, P. D., *J. Exp. Med.*, 1925, xlii, 99.
[5] Elman, R., and McMaster, P. D., *J. Exp. Med.*, 1925, xlii, 619.
[6] Fischler, F., Physiologie und Pathologie der Leber, Berlin, 1916.
[7] Whipple, G. H., *Physiol. Rev.*, 1922, ii, 440.

bacterial reduction of the bilirubin entering therein.[8-10] Certain bacteria only are capable of effecting this change, but several of such are present in abundance in normal dog feces.[9,11,12] When a dog is allowed to drain bile through an open fistula, as in the case of the animals used by previous workers, it is easy to see how the secretion and the biliary tract itself can become infected with these organisms, and how a change of bilirubin to urobilin can on occasion take place within the ducts, even when no pigment is entering the intestine. In our experiments already described[1-5] the biliary tracts occasionally became infected during the daily bile collection over long periods of time, despite the most careful precautions to maintain sterility. Saprophytic organisms, having no effect upon the pigment content of the secretion, were the usual contaminants. Rarely, however, the infected bile turned very cloudy, became pale orange in color, and gave off a foul odor. Urobilin could be recognized in it.

Present Knowledge.

Investigators have damaged the livers of animals subjected to total bile deprivation as result of open bile fistulas, and have found in a few instances, not in all, that large amounts of urobilin appeared in the bile and urine although the stools were acholic and urobilin-free.[6,7] It has been concluded from this finding that the damaged liver is capable of producing urobilin, some of which appears in the bile and some in the urine. In papers already published,[1-5] we have been able to show that when an animal deprived of bile ingests bile in any way,—and this would hold for one licking a biliary fistula,—the entrance of merely a little of the secretion into the intestine will have result in the appearance of urobilin in the bile put out by the liver. If at the same time there is liver injury the pigment will appear in the urine as well. Fischler[6] states that he has obtained urobilin in the bile and urine of animals when licking of the fistula was prevented, but the finding was obtained in but a few instances out of many studied. Here again it

[8] Müller, F., *Z. klin. Med.*, 1887, xii, 45.
[9] Kämmerer, H., and Miller, K., *Deutsch Arch. klin. Med.*, 1923, cxli, 318.
[10] Beck, A., *Wien. klin. Woch.*, 1895, viii, 617.
[11] Passini, F., *Wien. klin. Woch.*, 1922, xxxv, 217.
[12] Passini, F., and Czaczkes, J., *Wien. klin. Woch.*, 1923, xxxvi, 657.

seems probable that among many dogs with open fistulas and infected biliary tracts a few might carry within these tracts an organism reducing bilirubin to urobilin.

Method.

For our work healthy dogs were used, permanently intubated, in every instance, under ether anesthesia, so that all of the bile could be collected.[13] The methods for bile collection in 24 hour periods, and for the analysis of the bile specimens, urine, and feces, have been described in previous papers, together with the care and management of the animals.[1,2] At autopsy search was always made for evidences of reconstitution of the ducts and instances showing this were ruled out.

It so happened that one of the animals yielding a bile, that was consistently sterile for some months, became "spontaneously" infected with an organism that caused pigment changes in the bile as described above. Thus the secretion came to contain large amounts of urobilin although the feces and urine were urobilin-free. When sterile, urobilin-free bile specimens from other intubated dogs were inoculated, *in vitro*, with a few drops of this bile, enormous amounts of urobilin developed during 24 hours incubation. Attempts were made to isolate the causative organisms by plating out in an agar medium containing sterile bile, blood, and bouillon, but though several kinds of bacteria were isolated, these, when reinoculated into sterile bile either with or without the addition of protein, failed, singly or together, to effect the change to urobilin, although the bile became brown in color and cloudy.

The biliary tracts of 2 dogs giving sterile bile were now inoculated with 0.5 cc. of the original infected bile by retrograde injection through the intubation apparatus. The secretion obtained from the collecting balloon 24 hours later was foul, orange-colored, and contained much urobilin then and thereafter. The same result was attained in other dogs yielding sterile urobilin-free bile, by inoculating the biliary tracts with a suspension of their own fecal matter or that from normal dogs. The bile thus infected contained large amounts of urobilin and became cloudy, orange-colored, and foul.

13 animals intubated under ether for the collection of total bile were used in a study of the results of the infection. In 6 of them section of the cystic duct between ligatures and aspiration of the gall bladder had been carried out at the initial operation, while in the remaining 7 the gall bladder and its connections had been left undisturbed. Several days after the sterile bile had become urobilin-free, in consequence of the diversion of this secretion from the intestine, the biliary tract of each animal was deliberately infected. The infective material was often bile originally urobilin-free to which fecal particles had been added, or bile from an animal which had become "spontaneously" infected with result in the production of urobilin, or, frequently, bouillon suspensions of dog feces. The bouillon sus-

[13] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.

pension was employed on a single day to infect the biliary tracts of 2 animals with gall bladder connections undisturbed, and 2 in which the organ had been isolated by ligature and section of the cystic duct. In 4 other instances, 2 with gall bladder connections undisturbed and 2 with the organ isolated, the biliary tracts were inoculated on the same day with the bile of an animal that had become "spontaneously" infected in such a way that urobilin was formed in it. 6 days later bile of the same derivation was used to infect another animal with gall bladder connections undisturbed. Despite the identity of the biliary infections thus produced in the 2 groups of intubated animals the findings were distinctly different. That actual infection of the biliary tracts occurred, was ascertained by recovery of organisms therefrom at autopsy.

To inoculate the biliary tracts ½ to 3 cc. of the infective material was injected toward the liver into the tube leading to the cannula in the common duct. Thereafter all of the animals remained in good condition and the bile quantity put out each day continued to be about the same, but the secretion became cloudy, pale orange-brown, foul smelling, and urobilin-containing. The feces continued acholic and the urine remained free from urobilin. Quantitative estimations of the amount of this pigment in the bile were made daily, but, owing to the changes in the character of the secretion, quantitative determinations of bilirubin could no longer be carried out.

Urobilinuria Following Biliary Obstruction after Infection of the Biliary Tract.

Severe Urobilinuria When the Gall Bladder Connection Is Undisturbed.—The effect of biliary obstruction upon animals with infected biliary tracts but with the gall bladder left undisturbed will be described first. To produce obstruction the outlet tube leading from the common duct was clamped for periods of 48, or occasionally of 72, hours. 8 experiments of the kind were made upon 6 different animals. In 3 instances (No. 18, Table I, Text-fig. 1; No. 8, Table II, Text-fig. 2; and No. 17, Text-fig. 7) the experimental biliary obstruction was produced shortly after the deliberate infection of the biliary tract, and in 3 other animals at a time when complete recovery had ensued from liver injury due to administrations of chloroform and long after the production of the experimental infection (No. 12, Table III; No. 14, Table IV; and No. 10, Text-fig. 6). In all these experiments bilirubinuria developed. The effect of the obstruction can be roughly estimated from its degree. A pronounced urobilinuria developed following the obstruction and frequently more urobilin than bilirubin was voided through the kidneys despite the absence from the

intestine of bile, which is of course the normal source of urobilin.[2] Following the relief of obstruction the urobilin and bilirubin of the urine disappeared together.

In 2 other experiments total biliary obstruction was produced after chronic infection had produced pathological changes in the gall bladder. One of these experiments, the results of which are shown in the final portion of Text-fig. 7, was done after an obstruction experiment, the other, Table III, after recovery from liver damage by chloroform and a later biliary obstruction. These complicated instances will be considered in detail further on. In neither could a pronounced urobilinuria be elicited.

Specimen Protocols.

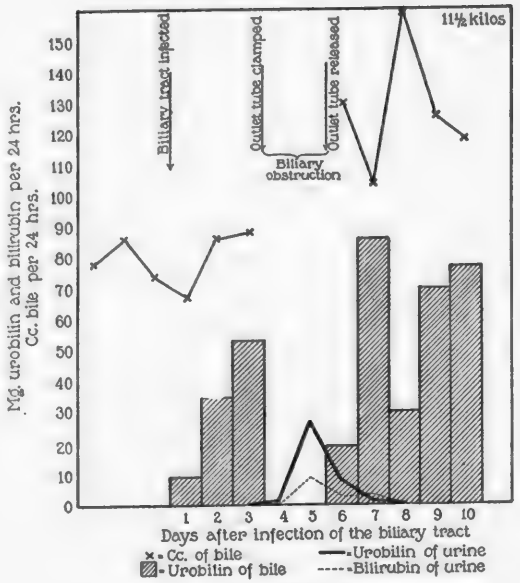
No. 18, 11½ kilos (see Table I, Text-fig. 1).

Simple intubation of the common duct was done under ether. 5 days later the animal was secreting sterile, urobilin-free bile and the urine and feces were also urobilin-free. Now the biliary tract was infected by injecting into the tube leading to the common duct, 3 cc. of a suspension of fecal particles in broth. By means of a syringe this was gently forced up into the ducts through the rubber tube connected with the common duct and held there for a moment, after which the pressure was released and bile flow allowed to continue as usual. Thereafter the 24 hour bile specimens regularly contained large quantities of urobilin (9, 35, and 53 mg., respectively). During this period the urine and feces remained free from bile pigments.

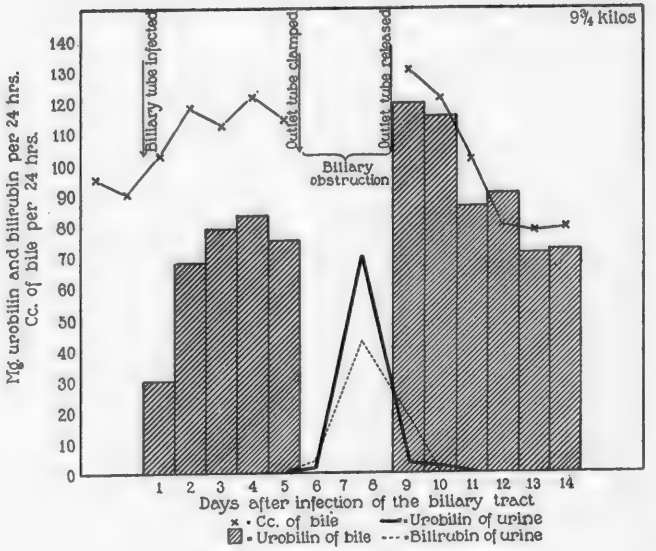
Biliary obstruction was next produced for a period of 48 hours by clamping the outlet tube from the common duct. The 24 hour specimen of urine collected the following day contained both urobilin and bilirubin, more of the former than of the latter. In the urine of the second 24 hour period of biliary obstruction there was a far greater quantity of urobilin (27 mg.) than of bilirubin (9.0 mg.). With the relief of the obstruction both pigments disappeared from the urine in a short time, as the chart shows.

No. 8, 9¼ kilos (see Table II, Text-fig. 2).

About 1 month after intubation of the common duct under ether the animal, in excellent condition, was secreting sterile urobilin-free bile. The urine and feces were acholic and also urobilin-free. The biliary tract was then infected by injecting into the tube leading to the common duct, 0.5 cc. of the bile of Dog 3 already infected with urobilin-forming organisms. This was pressed up toward the ducts and the pressure at once relieved and bile flow allowed to continue. The 24 hour bile specimens taken as usual contained, on the 5 successive days after this inoculation, great amounts of urobilin, 30, 68, 79, 83, and 75 mg. The bile volume put out was practically constant.



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIGS. 1 and 2. Marked urobilinuria consequent on biliary obstruction in dogs that had been draining a bile experimentally infected with urobilinproducing organisms. The gall bladder connection with the common duct had been left undisturbed at the time of the intubation.

Biliary obstruction for a period of 72 hours was next produced by clamping the outlet tube leading to the collecting balloon from the common duct. The urine of the following 24 hours showed but little urobilin and bilirubin, 2 mg. of the former and 3.5 mg. of the latter. The next day no urine was voided; but the specimen of the following 24 hour period contained, in 350 cc., 70 mg. of urobilin and 42 mg. of bilirubin. With the relief of biliary obstruction the urobilin of the urine fell almost at once to about 3 mg. and 48 hours later had disappeared altogether. The bilirubin, though lessening not quite as rapidly in the first 24 hours after the relief of the obstruction, disappeared nevertheless slightly before the urobilin.

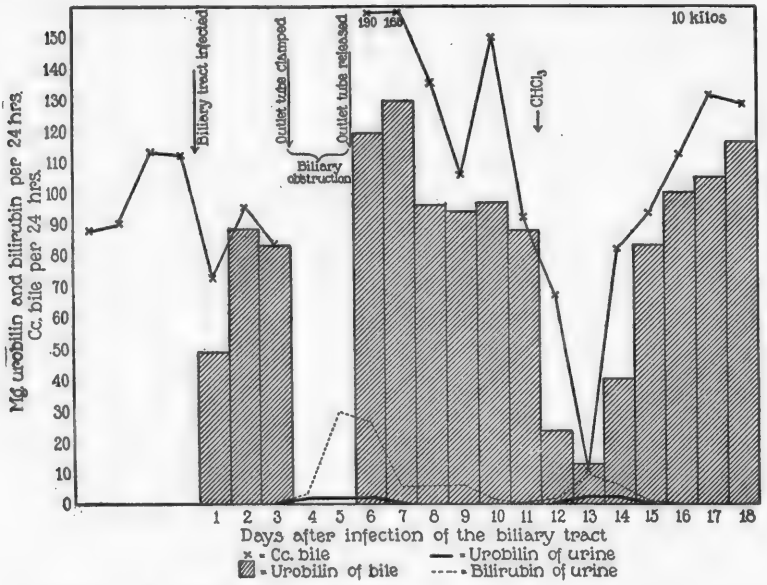
In all the remaining animals of this series pronounced urobilinuria developed after obstruction to the bile flow, following in turn the infection of the biliary tract. In Tables I and II and in Text-fig. 7, are represented the findings in the 3 animals subjected to biliary obstruction in this way. In the latter portions of Tables III and IV and in Text-fig. 6, similar findings are shown in other experiments of this kind, made after the animal's readjustment to injury of the liver by chloroform.

After biliary obstruction, then, in animals with gall bladder connections undisturbed and with the biliary tract infected with an organism causing urobilin formation, quantities of this pigment appeared in the urine. This occurred even though no bile had reached the intestine for weeks, during which period no urobilin was formed in the normal way.

Trifling Urobilinuria When the Cystic Duct Has Been Severed.—The findings were quite other when the connection of the gall bladder with the biliary tract had been interrupted by ligation and section of the cystic duct. Under these circumstances not only can the gall bladder no longer function, but in the absence of infection it diminishes in size as is well known[14] and its contents become pale and mucoid. In such instances urobilin appeared in the urine when an obstruction of the appropriately infected biliary tract was produced, but only in traces. 7 experiments of the sort were made upon 6 different animals and this was the finding in all. In 3 of these experiments total biliary obstruction for 48 hours was produced soon after the biliary tract had

[14] Mignot cited by Aschoff and Bacmeister who confirm the observation. Aschoff, L., and Bacmeister, A., Die Cholelithiasis, Jena, 1909.

been infected and later, when the animal had recovered, liver damage was caused by inhalations of chloroform (see Text-fig. 3; and also Tables VI and VII). In 3 further experiments the procedures were reversed (see Tables VIII and IX; and also Text-fig. 4). The findings in them were similar.


10 kilos
Biliary tract infected
Outlet tube clamped
Outlet tube released
Biliary obstruction
CHCl3
Mg urobilin and bilirubin per 24 hrs.
Cc. bile per 24 hrs.
Days after infection of the biliary tract
× = Cc. bile
▨ = Urobilin of bile
— = Urobilin of urine
---- = Bilirubin of urine

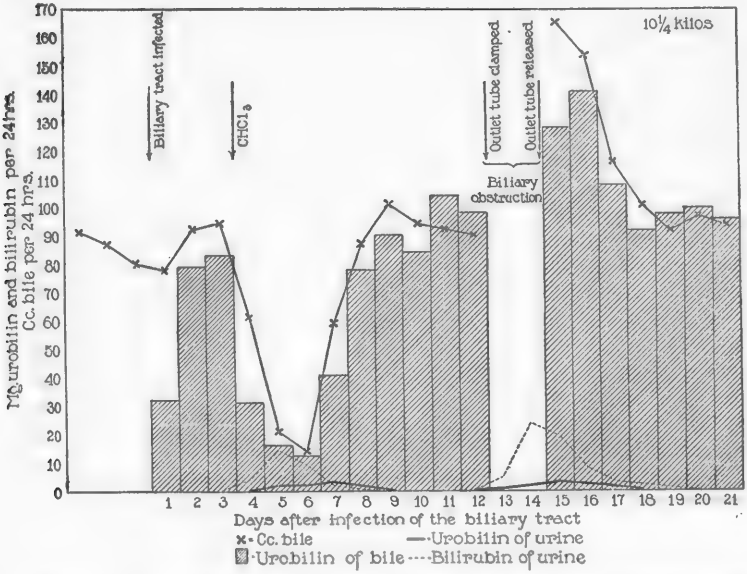
TEXT-FIG. 3. Urobilinuria consequent on a temporary biliary obstruction and again after liver injury with chloroform, occurring in an animal draining a bile experimentally infected with urobilin-producing organisms. The gall bladder connection had been destroyed by ligature and severance of the cystic duct. The urobilinuria is neglible as compared with that of Text-figs. 1 and 2.

Specimen Protocol.

No. 5, 10 kilos (see Text-fig. 3).

2½ months after intubation of the common duct under ether and section of the cystic duct, the biliary tract of this dog was inoculated with the same infectious material employed to infect the biliary tracts of Animals 11, 8, and 13 and on the same day. The daily amount of bile secreted afterwards by this animal contained great quantities of urobilin, though previous to the infection the pigment had been absent. The urine and feces were urobilin-free. Biliary obstruction was effected

3 days later by clamping the outlet tube for a period of 48 hours. Slight bilirubinuria and urobilinuria developed within 24 hours, as the text-figure shows. During the second 24 hours of obstruction the amount of bilirubin in the urine increased to 29 mg., but urobilin was found in traces only, never more than 2 or 3 mg. Following the relief of obstruction both pigments disappeared, the bilirubinuria enduring for several days however. Liver injury was now produced by chloroform administrations. The findings in these experiments will be considered in detail further on.



TEXT-FIG. 4. This shows the same facts as Text-fig. 3; but in this instance the chloroform injury preceded the induced biliary obstruction.

Text-fig. 4 is the record of an experiment similar to the above following recovery of the animal from liver damage by chloroform (Dog 11).

12 days after infection of the previously sterile biliary tract with the material used for the experiments with Dogs 5, 8, and 13, and 20 days after intubation of the common duct with section and ligature of the cystic duct under ether anesthesia, total biliary obstruction was produced. The animal had recovered from the effects of a prior chloroform administration and the bile and urine were urobilin-free. As the latter portion of Text-fig. 4 shows, the bile still contained large amounts of urobilin, 84, 104, and 98 mg., respectively, on the 3 days preceding the obstruction. As the chart further shows 48 hours of biliary obstruction

resulted in severe bilirubinuria, 23.7 mg. of the pigment appearing in the urine during the second 24 hour period thereafter. The bilirubinuria decreased after relief of the obstruction, to disappear 6 days later. Urobilinuria also made its appearance following the experimental biliary obstruction, but in contrast to the findings in animals with the gall bladder connections undisturbed, only very small amounts of the pigment were present in the urine. On the 4th day after relief of the obstruction the pigment was no longer present there. In Tables VI to IX and in Text-figs. 3 and 4 are given the results of the experiments.

The precise character of the infection would seem to have no importance in connection with the difference here brought out between animals with and without a functioning gall bladder. Several illustrations of this fact can be given:

3 dogs with severed cystic duct, were inoculated on the same day with bile from Dog 3 which had become "spontaneously" infected with a mixed growth of bacteria capable of producing urobilin out of the bilirubin. In one of these animals No. 5 (Text-fig. 3), as well as in Dog 3 itself (Table VII) the experiments involving biliary obstruction were followed after an interval by chloroform administrations. In another of the animals so infected, Dog 11 (Text-fig. 4), the procedures were reversed with similar results. This same infective material was used on the same day to infect the biliary tracts of 2 other dogs of the first series, animals that is to say with undisturbed gall bladder connections. In one of these, No. 8 (Table II, Text-fig. 2), biliary obstruction was followed after recovery by administrations of chloroform; in the other, Dog 13 (Table V), the only derangement induced was liver damage from chloroform. Although the bile of all these dogs was infected in the same way the urobilin findings differed in the 2 series as will be described below. Other instances of differing results in the 2 groups of animals, despite the fact that the infective material was the same, are to be found in Tables I and IV, and in Text-fig. 7, of instances with the gall bladder connections undisturbed, and in Tables VIII and IX, of animals with severed cystic ducts.

Urobilinuria Following Liver Damage after Infection of the Biliary Tract.

Pronounced Urobilinuria When the Gall Bladder Connection Is Undisturbed.—After the urine of some of the intubated animals with undisturbed gall bladder connection had become urobilin- and bilirubin-free and when the daily bile specimens contained large quantities of urobilin as result of infection, liver injury was brought about by chloroform.

Chloroform anesthesia is followed within 48 hours by a reduction

in the output of bile and bile pigment, with a consequent development of bilirubinuria.[4,5,15] The degree of liver damage can be roughly estimated by the lessened bilirubin of the bile or by the amount of it appearing in the urine. The latter gauge was used in the present instance owing to the impossibility of accurately quantitating the bilirubin of the infected biles.

The chloroform injury led in each instance to a marked urobilinuria, although there was relatively slight liver damage, as shown by the slight bilirubinuria. 2 of the experiments were made upon animals with infected biliary tracts, and with a bile containing urobilin, following readjustment from a temporary biliary obstruction (Tables I and II). In 4 other animals with infected biliary tracts the effects of liver damage by chloroform upon the output of urobilin in the urine were studied prior to the production of biliary obstruction (Table III, Text-fig. 5, Tables IV and V, and also Text-fig. 6). In the instance last mentioned a second administration of chloroform 34 days after the first elicited but a slight result because pathological changes had taken place in the gall bladder. This phenomenon will be discussed below.

In Text-fig. 5 the findings are charted of 1 of the experiments in which chloroform was administered soon after infection of the biliary tract and prior to obstruction. These findings are typical.

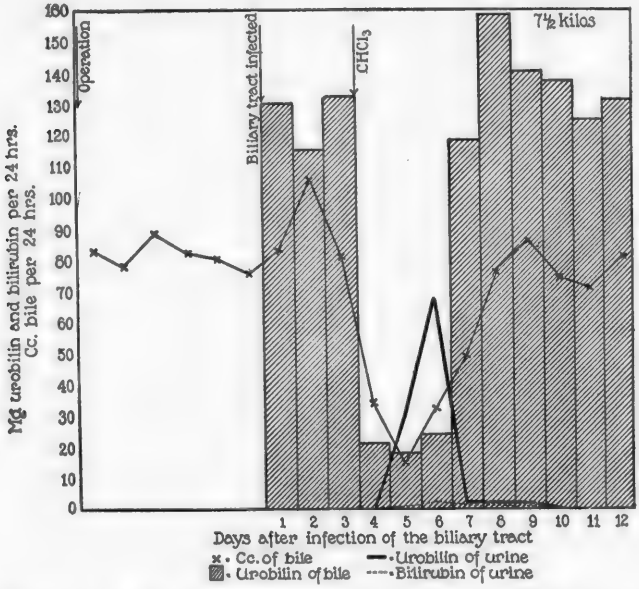
Specimen Protocol.

No. 12, 7½ kilos (see Table III, Text-fig. 5).

Intubation of the common duct was done under ether anesthesia, and 6 days later the urine and feces had become urobilin-free. 3 days later the biliary tract was infected by injecting into the tube leading to the common duct, 0.2 cc. of a fresh suspension of fecal material from the dog, in 3 cc. of sterile broth. The material was gently forced up toward the ducts and immediately thereafter the bile was allowed to flow again toward the collecting balloon. The specimens of the next 3 days contained 130, 115, and 132 mg. of urobilin, respectively. Chloroform anesthesia was now induced for a period of 30 minutes, 60 cc. being employed. The next day, as the chart shows, the bile volume fell to less than half its previous amount and remained smaller than usual for 3 days. The urobilin of the secretion also decreased to about 20 mg. for each 24 hour period. 48 hours after the anesthesia, 29.5 mg. of urobilin was present in the urine, and a day later 67.5 mg. The bilirubinuria

[15] Drury, D. R., and Rous, P., *J. Exp. Med.*, 1925, xli, 611.

during this period was but slight, varying between 1.0 and 2.0 mg. for each 24 hours. This finding, together with the fact that complete bile suppression did not occur, shows clearly that the actual liver damage produced by the half hour of anesthesia was relatively slight, as would be expected. Recovery was prompt; and on the 5th day after the chloroform administration the usual secretion of bile had taken place. The urobilin content of the bile returned to the previous level and subsequently rose even higher. Simultaneously with the recovery the urobilinuria and bilirubinuria promptly decreased until only the merest traces of pigment were to be found, and 2 days later these had disappeared altogether.



TEXT-FIG. 5. Marked urobilinuria consequent on liver damage by chloroform in a dog draining a bile infected with urobilin-producing organisms. The gall bladder connection had not been disturbed.

For the sake of brevity no other protocols of similar experiments will be given. In Tables III, IV, and V the findings will be found tabulated however, and in Tables I and II the results are given of chloroform administrations to other dogs with gall bladder connections undisturbed, after their readjustment to the effects of biliary obstruction. The findings in all were similar, save for one instance, already mentioned, in which after infection had endured 5 weeks and

the gall bladder had become pathologically changed, no pronounced urobilinuria was elicited by liver damage. In this animal, however, (Text-fig. 6) chloroform injury, 3 days after infection of the biliary tract, produced a result similar to those described above.

Trifling Urobilinuria after Exclusion of the Gall Bladder.—When experiments similar to the ones just described were made upon animals infected in the same way, but with the cystic duct cut between ligatures, a striking difference was noted. Liver damage by chloroform again produced urobilinuria, but a very mild one. Only the merest traces of the pigment were present in the urine in spite of the fact that the liver injury was sufficient in some instances to bring about severe bilirubinuria. 6 animals were given sufficient doses of chloroform to produce marked liver damage. In 3 of the dogs (Tables VI and VII and Text-fig. 3) this was done after recovery from experiments involving biliary obstruction, and in 3 others prior to this (Tables VIII and IX, and Text-fig. 4). The results were the same in all instances. A protocol of each type of experiment follows:

No. 5, 10 kilos (see Text-fig. 3).

2½ months after intubation of the common duct with ligation and section of the cystic duct under ether anesthesia, infection of the biliary tract was effected, and biliary obstruction was produced later. 6 days after the relief of this the urine and feces had become urobilin-free. The infected bile contained much of the pigment. At this time, 11 days after the infection of the biliary tract, liver damage was produced by chloroform anesthesia for 40 minutes, 65 cc. of chloroform being used. On the following day, as Text-fig. 3 shows, the bile volume fell and so too did its urobilin content. The 24 hour urine specimen contained but 1.8 mg. of bilirubin and no urobilin. The next day, the 2nd after the anesthesia, still less bile was put out. Urobilin appeared in the urine, 2.5 mg. in the 24 hour specimen, while there was much more bilirubin, 9.6 mg. On the following day improvement began to take place, the bile output increased, and the bilirubinuria decreased. Urobilin continued to be found in the urine, but with further improvement in the animal's condition disappeared entirely 24 hours later.

The result of a similar experiment, carried out before biliary obstruction was attempted, is shown in Text-fig. 4, the liver injury leading merely to traces of urobilin in the urine despite the fact that it was sufficient to bring about a moderate bilirubinuria.

No. 11, 10¼ kilos (see Text-fig. 4).

An intubation, together with section of the doubly ligated cystic duct, was

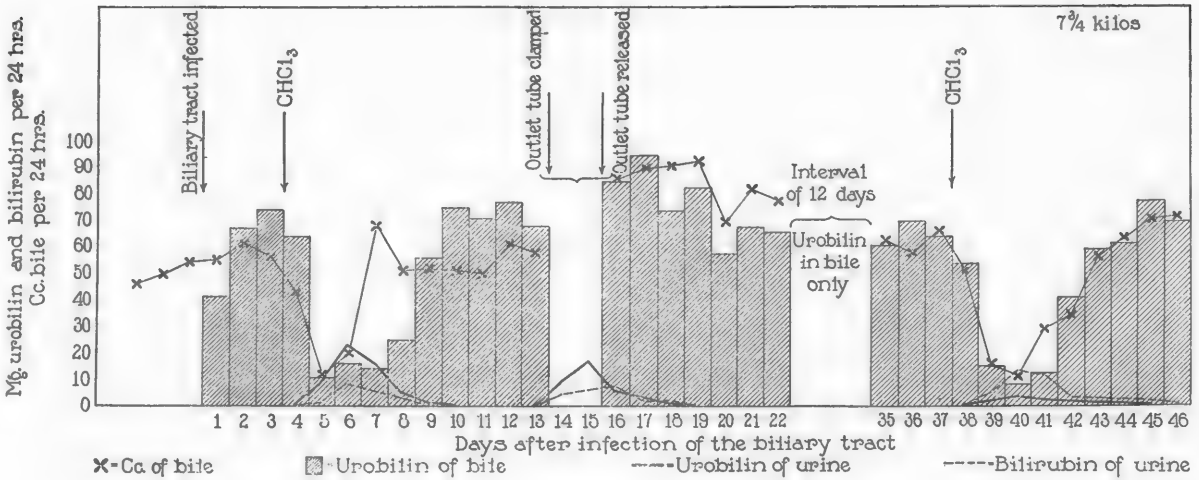
done under ether. 6 days later the bile, urine, and feces were urobilin-free. 2 days after this the biliary tract was infected with the same material used to infect the biliary tracts of Dog 5 (Text-fig. 3), Dog 8 (Table II, Text-fig. 2), and Dog 13 (Table V) and on the same day. Urobilin appeared in the bile within 24 hours. 3 days later chloroform was administered. Immediately, as the chart shows, the bile volume fell, until on the 3rd day it was but one-fifth its former value. In the meantime a marked bilirubinuria developed reaching its maximum of 14 mg. of the pigment in the second 24 hour period after the chloroform anesthesia, to disappear entirely 4 days later, after recovery had taken place. Though bilirubin was present in the urine the day after chloroform was given, urobilin did not appear until next day, and then small amounts only were found, 2 or 3 mg. per 24 hours, on 4 successive days, disappearing from the urine with the animal's recovery.

In Text-fig. 4 are shown further the effects of biliary obstruction upon this animal.

From these experiments it can be said that, in dogs with cystic ducts ligated and sectioned to prevent the activity of the gall bladder, and with the biliary tracts infected in such a way that urobilin is formed from the bilirubin of the bile, liver damage, whether slight or severe, is attended only by trifling amounts of urobilin in the urine. The findings afford a striking contrast to those of the experiments in which the gall bladder connections were undisturbed.

Failure of Marked Urobilinuria to Appear When Chronic Infection Had Caused Pathological Changes in the Gall Bladder.

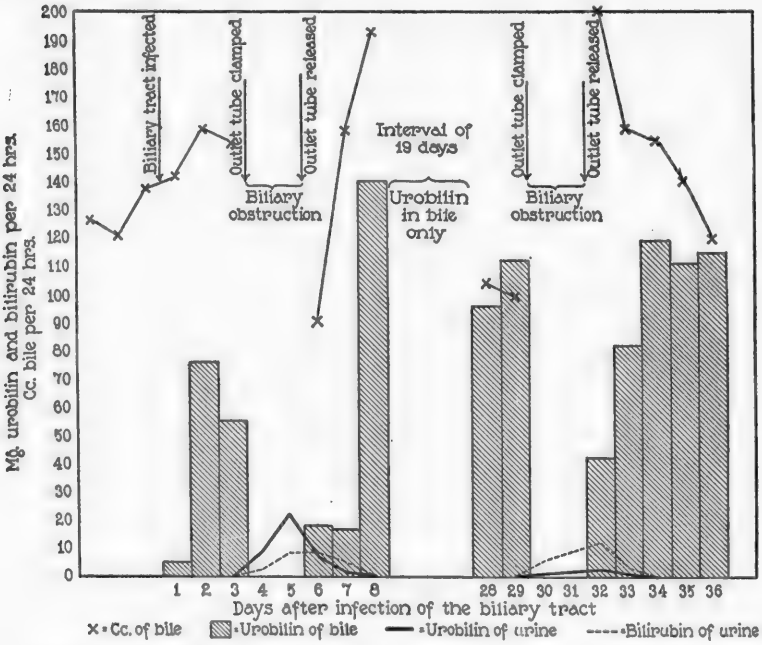
In such animals with gall bladder connections intact, as excreted large amounts of urobilin in the urine during the experiments, the organ appeared healthy at autopsy and contained a concentrated, infected bile. In several dogs, however, the marked urobilinuria produced shortly after infection of the biliary tract by damage to the liver with chloroform, and produced again after recovery from this by an experimental obstruction, could not be elicited 2 weeks or more later by the same experimental means, only traces of the pigment then appearing in the urine. Autopsy in such cases (Text-fig. 7, Text-fig. 6, and Table III) showed gall bladders that had undergone pathological changes as result of infection with a thickening of the wall. The contents of the viscus was no longer a more or less concentrated bile as when it is healthy, but light in color and mucoid.



TEXT-FIG. 6. **Failure of marked urobilinuria to occur under the conditions which would have led to it had not the infection with urobilin-producing organisms caused pathological changes in the gall bladder.**

No. 10, 7¾ kilos (see Text-fig. 6).

7 days after an intubation under ether anesthesia, with gall bladder connections undisturbed, urobilin had disappeared from bile, urine, and feces. The biliary tract was then inoculated with the infected bile of Dog 3 which had been employed 6 days previously to inoculate the biliary tracts of Dogs 5, 8, 11, and 13. On the day following, urobilin was present in the bile and it was constantly found there later.



TEXT-FIG. 7. This shows the same facts as Text-fig. 6, but in this instance biliary obstruction only was induced.

Moderate liver damage from chloroform 3 days after infection of the biliary tract brought about marked urobilinuria as is shown in the chart. Shortly after the animal's recovery, a marked urobilinuria was again produced by biliary obstruction. Following relief of it 2 weeks passed, during which period urobilin was to be found in the bile only.

Liver injury was brought about again by inhalations of chloroform, after the biliary infection had endured 5 weeks, and the injury was more severe than in the first instance, as shown by a greater amount of bilirubin in the urine. In spite of

this, however, urobilin appeared in small amounts only in the urine. Immediately after the animal's recovery autopsy was done. A greatly changed gall bladder was found, with a much thickened wall. The viscus contained a pale yellow, cloudy, mucoid fluid.

No. 17, 12 kilos (see Text-fig. 7).

The marked urobilinuria caused by biliary obstruction, 3 days after infection of the biliary tract with the material that was used as well for Dogs 14, 15, 16, and 18, is shown in the chart. The gall bladder had not been disturbed. 3 days after relief of the biliary obstruction the urine and feces became urobilin- and bilirubin-free. 21 days were then allowed to elapse, during which daily bile specimens were taken and found to contain large quantities of urobilin. The urine and feces still remained urobilin-free. Now, 29 days after the initial infection, obstruction of the flow of bile was again brought about by clamping the outlet tube from the common duct. As the chart shows, as marked a bilirubinuria developed as before, testifying that the obstruction was complete; yet the urine contained far less urobilin than on the previous occasion. The chart is like that of an animal with obstructed cystic duct. Autopsy 3 days after the urine became urobilin-free disclosed a gall bladder with pale yellow, cloudy, mucoid contents and pathological changes in its greatly thickened wall.

DISCUSSION.

The experiments here described throw light upon the mechanism of a type of urobilinuria previously considered due to the formation of urobilin from the liver cells. Instead of invoking such a parenchymal activity—for which our extensive experiments already reported[1–5] yield not the slightest evidence—it is now possible to explain the observed facts by the absorption of urobilin produced through bacterial action on the bile passing through ducts and gall bladder.

Granting that urobilin is formed on occasion within appropriately infected bile ducts, as our experiments show, and that when total obstruction is produced or the liver is otherwise injured, urobilinuria occurs, how is one to suppose the pigment to be absorbed? It is significant that the pronounced urobilinuria occurred only in the animals with intact gall bladders. In these instances there is of course a larger amount of bile in the biliary tract to be acted upon by the bacteria. There is further more opportunity for their activities since the bile remains long within the tract; and the secretion is exposed to a larger surface from which absorption may conceivably take place, and to the activities of an organ specifically engaged in concentration

of the bile. As result of these conditions more urobilin is formed and there is more of it to be removed into the circulation. While urobilin is known to be a highly diffusible substance direct proof has yet to be produced of its absorption through the gall bladder wall. Certain it is however that more urobilin reached the urine in our animals with healthy gall bladders than in those in which this organ had been injured or excluded from connection with the ducts. Experiments on the activity of the gall bladder to remove urobilin from the bile are contemplated for the future.

The possible relation of infection to the presence of urobilin in the bile has long been discussed and its etiological relationship to urobilinuria has been surmised but not demonstrated. The importance of the factor of infection is made plain by the present work, especially when this is considered in connection with the previous papers of the series.[1–5] In these papers it has been shown that when bile pigment is prevented from reaching the intestine and the biliary tract is uninfected no urobilin is produced in the body even though the liver be severely injured or much blood destroyed. If small amounts of bile pigment are allowed to reach the intestine, *per os* or otherwise, urobilin is formed and appears in the bile. Normally the urobilin, formed in the intestine by the bacterial reduction of bilirubin, is absorbed and removed from the blood by the liver. When this organ has been injured the pigment appears in the urine, owing to failure of the hepatic tissue to prevent the passage of urobilin into the general circulation.

Investigators have produced liver injury in dogs[6, 16] and found urobilin in the urine as a result. The finding has been ascribed to a perversion of pigment metabolism by the liver cells in consequence of which they form urobilin. But in all experiments of this nature an open, infected fistula has been present, one infected doubtless with organisms capable of producing urobilin in the bile. Such organisms are present in dog feces and the animal usually has access to the fistula opening. We believe that all of the instances of the supposed hepatic formation of urobilin may be explained in this way.

The fact should be emphasized that only a certain type of infection will bring about the changes described. The biliary tract may be

[16] Kunkel, A., *Virchows Arch. path. Anat.*, 1880, lxxix, 455.

infected for a long time with the ordinary saprophytes or even with pyogenic organisms without any reduction of the bilirubin to urobilin. Some fecal bacteria by contrast are capable of maintaining themselves in the biliary passages for indefinite periods of time, there causing urobilin formation. That some of the urobilin is absorbed our experiments show. In draining bile through an open fistula it is easy for infection to take place. Such an occurrence with urobilinuria developing as a result of liver injury has been noted by Wallace and Diamond.[17]

The question whether a manufacture of urobilin ever takes place within the biliary tract of human beings other than those having biliary fistulas is one of much clinical importance. A bacteriological study of infected biliary tracts should be undertaken by surgical pathologists with a view to determining the incidence of urobilin-producing organisms.

Very recently Fischler and Ottensooser[18] have reported what they consider to be an hepatic formation of urobilin. Their experiments were carried out upon the rabbit and involved the production of biliary obstruction followed by "glycopriven intoxication" for the purpose of injuring the liver. Urobilinuria developed. The animals became heavily jaundiced of course and under these conditions an enterogenous origin of urobilin can never be excluded. For it is well known that in the presence of tissue icterus the intestinal mucous membrane becomes deep yellow and bilirubin is put out into the lumen of the bowel.[19,17] As we have already shown, biliary obstruction in the dog is followed by temporary disappearance of urobilin in the feces[2] but when tissue icterus develops the pigment reappears therein. Wallace and Diamond[17] have shown that under such conditions urobilin is formed on the outer layers of the fecal masses in contact with the intestinal walls. Fischler and Ottensooser found no urobilin in the feces of their animals after the production of biliary obstruction but this does not exclude the possibility of its formation in the intestine with subsequent complete reabsorption, or partial absorption of it and destruction of the remainder. We have frequently found urobilin appearing suddenly in the bile of dogs which had previously been urobilin-free after diversion of the bile from the intestine. The stools of such animals remained urobilin-free but at autopsy reconstitution of the bile ducts was found permitting a small quantity of bile to reach the intestine. The urobilin formed from this was absorbed and reappeared in the bile, or, if liver injury was

[17] Wallace, G. B., and Diamond, J. S., *Arch. Int. Med.*, 1925, xxxv, 698.
[18] Fischler, F., and Ottensooser, F., *Deutsch Arch. klin. Med.*, 1925, cxlvi, 305.
[19] Gerhardt, D., *Z. klin. Med.*, 1897, xxxii, 303.

produced, in the urine, but the feces remained urobilin-free. Such a urobilinuria would still be enterogenous in origin.

In the experiments under discussion these authors frequently found urobilin in the contents of the gall bladders of their rabbits. Whatever the source of the pigment it is easy to see from the findings given in the present paper that it would appear in the urine of the animals after the liver injury caused by the "glycopriven intoxication."

We believe that pigment studies involving biliary obstruction in the rabbit are inevitably dubious in nature, for this animal secretes relatively enormous quantities of bile and obstruction to its flow results in a widespread immediate necrosis of the liver with profuse secondary cirrhosis. The rabbit like the other herbivora secretes a bile containing pigments unlike those of man or the dog. All this being the case when rabbits are put in a state of extreme hypoglycemia with as result a liver damage in addition to that from obstruction, as happened in Fischler's experiments, almost any anomaly in pigment metabolism might occur.

In previous papers Fischler[6] has concluded that a production of urobilin through the action of the liver parenchyma occurs under special conditions. He had found that urobilin is present in the bile of dogs draining from open fistulas after the liver had been injured with amyl alcohol and phosphorus. The fact is readily to be explained by the presence of an infection of the biliary tract with urobilin-producing bacteria which latter have more time to act upon the bile when it flows sluggishly, as after liver injury. Whipple believes that the occurrence of urobilin in the bile of dogs with open fistulas when such animals are fasted comes about in this way.[20]

The results of the experiments here described serve to confirm those of our previous papers, which show that there is no formation of urobilin by the hepatic parenchyma. The fact is to be emphasized that urobilin appears only when the bilirubin of the bile is subjected to reduction by certain bacteria. Normally this takes place in the intestine but it may also occur in a biliary tract contaminated with the appropriate organisms. Urobilin in the urine depends, first upon the absorption of the substance from these situations, and secondly, upon the failure of the liver to remove the pigment from the blood.

SUMMARY.

Experimental infection of the intubated and previously sterile biliary tract of the dog with particles of the stools leads to a formation

[20] Whipple, G. H., Hooper, C. W., and Robscheit, F. S., *Am. J. Physiol.*, 1920, liii, 167.

of urobilin from the bilirubin of the bile as it flows through the ducts. No urobilinuria occurs, however, unless temporary biliary obstruction is produced, or the liver parenchyma injured. Then urobilinuria develops, despite the fact that no bile is reaching the intestine and, by corollary, no urobilin being formed there.

Cholangitic urobilinuria, as one may term the phenomenon just described, to distinguish it from the urobilinuria having origin in pigment absorbed from within the intestine, is far more pronounced in animals possessing a healthy gall bladder than in those with a pathological gall bladder or with one prevented from functioning by severance of the cystic duct. These facts suggest that there may be an active absorption of urobilin from the normal gall bladder. There can be no doubt that the pigment is absorbed from within the bile ducts.

There is no evidence whatever to justify the belief that urobilin is ever formed through the action of liver parenchyma. There may conceivably be an intralobular formation of the pigment consequent upon the activity of bacteria within the liver tissue, though such a happening has yet to be demonstrated.

TABLES I AND II.

Pronounced Urobilinuria Occurring in Animals with Gall Bladder Connections Undisturbed and an Infected Biliary Tract. The Pigment Appeared in the Urine When Biliary Obstruction Was Produced and Again Later When the Liver Was Injured with Chloroform.

TABLE I.

Dog 18.

Period after Operation.	Period after Infection of the biliary tract.	Amount of bile.	Total urobilin in bile.	Total urobilin in urine.	Total bilirubin in urine.	Weight.	Remarks.
days	*days*	*cc.*	*mg.*	*mg.*	*mg.*	*kg.*	
Intubation. Gall bladder undisturbed. 4 days later.							The findings of the first 13 days of this experiment are charted in Text-fig. 1.
4		85	0	Tr.	0	11	
5		74	0	0	0		
Biliary tract deliberately infected.							The infective material, a bouillon suspension of dog fecal particles, was employed on the same day to infect Dogs 14, 15, 16, and 17.
6	1	67	9	0	0		
7	2	86	35	None voided.			
8	3	88	53	0	0	11	
Total biliary obstruction produced.							Biliary obstruction induced prior to the liver injury experiment.
9	4			2	Tr.		
10	5			27	9		
Obstruction relieved.							
11	6	130	19	7.5	2.6		
12	7	104	86	F. tr.	3.5		
13	8	161	30	0	0		
14	9	126	67	0	0		
15	10	107	74	None voided.			
16	11	116	72	0	0		
17	12	103	62	0	0		
18	13	98	56	0	0	10½	
Chloroform anesthesia 40 min.							Infection had endured 2 wks. Pronounced urobilinuria developed following liver injury.
19	14	55	44	D.tr.	D.tr.		
20	15	12	11	21.5	4.1		
21	16	13	8	28.6	7.7		
22	17	31	19	8.4	8.8		
23	18	67	40	3.1	5.9		
24	19	72	58	Tr.	2.5		
25	20	117	73	0	1+	10½	At autopsy, 28 days after incubation and 23 days after infection of the biliary tract, a normal gall bladder was found, with dark brown, concentrated contents.
26	21	104	68	None voided.			
27	22	99	52	0	0		
28	23	108	61	0	0	10½	

In the tables Tr. indicates trace; F. tr., faint trace; and D. tr., dubious trace.

TABLE II.

Dog 8.

Period after Operation.	Period after Infection of the biliary tract.	Amount of bile.	Total urobilin in bile.	Total urobilin in urine.	Total bilirubin in urine.	Weight.	Remarks.
days	*days*	*cc.*	*mg.*	*mg.*	*mg.*	*kg.*	
Intubation. Gall bladder undisturbed. 36 days later.							The findings of the first 17 days of this experiment are charted in Text-fig. 2.
36	St.	90	0	0	0		
37	"	65	0	0	0	$9\frac{3}{4}$	
38	"	95	0	0	0		
39	"	89	0	0	0		
Biliary tract deliberately infected.							The infective material, bile of Dog 3, used to inoculate the biliary tract of this animal, was employed on the same day to infect Dogs 5, 11, and 13, and 6 days later Dog 10.
40	1	103	30	0	0		
41	2	118	68	0	0		
42	3	112	79	0	0		
43	4	121	83	0	0		
44	5	114	75	0	0	$9\frac{3}{4}$	
Total biliary obstruction.							Biliary obstruction induced prior to the liver injury experiment.
45	6			2	3.5		
46	7			None voided.			
47	8			70	42		
Obstruction released.							
48	9	130	119	3	19		
49	10	121	115	2	0		
50	11	101	86	0	0		
51	12	80	92	0	0		
52	13	78	73	0	0		
53	14	79	75	0	0	9	
Chloroform anesthesia 40 min.							Infection had endured a little over 2 wks., nevertheless there developed a pronounced urobilinuria following injury to the liver.
54	15	54	68	0	Tr.		
55	16	23	22	19	5.5		
56	17	18	14	25	9.5		
57	18	26	51	21	9.2		
58	19	38	54	6	3.2		
59	20	62	84	2.5	1+	$8\frac{3}{4}$	Autopsy 62 days after intubation and 23 days after infection of the biliary tract disclosed a healthy gall bladder partly filled with concentrated bile.
60	21	82	107	0	0		
61	22	86	92	0	0		
62	23	80	86	0	0	$8\frac{3}{4}$	

St. indicates sterile.

TABLE III.

Pronounced Urobilinuria Occurring in an Animal with Gall Bladder Connections Undisturbed and an Infected Biliary Tract. Large Amounts of the Pigment Appeared in the Urine after Liver Injury by Chloroform and Again Later When Biliary Obstruction Was Produced. The Effect of Obstruction in a Late Stage of Infection When Pathological Changes Had Occurred in the Gall Bladder Is Also Shown.

Dog 12.

Period after Operation.	Period after Infection of the biliary tract.	Amount of bile.	Total urobilin in bile.	Total urobilin in urine.	Total bilirubin in urine.	Weight.	Remarks.
days	*days*	*cc.*	*mg.*	*mg.*	*mg.*	*kg.*	
Altercursive intubation Gall bladder undisturbed. 6 days later.							The findings of the first 15 days of this experiment are charted in Text-fig. 5.
6	St.	82	0	0	0	7½	
7	"	80	0	0	0		
8	"	75	0	0	0		
Biliary tract deliberately infected.							The infective material used consisted of a bouillon suspension of particles of the animal's clay-colored feces.
9	1	83	130	0	0	7½	
10	2	105	115	0	0		
11	3	81	132	0	0	7¼	
Choloroform anesthesia 30 min.							Liver injury experiment performed prior to the induction of biliary obstruction.
12	4	34	21	0	0		
13	5	15	18	29.5	0.56		
14	6	32	24	67.5	2.0		
15	7	49	118	2+	1.75		
16	8	76	158	2	1.47		
17	9	87	140	2	1.15		
18	10	74	137	0	0	6½	
19	11	70	119	0	0		
20	12	79	125	0	0		
21	13	82	118	0	0	6¾	
Total biliary obstruction.							Infection of the biliary tract had endured 2 wks. Nevertheless there developed a pronounced urobilinuria following biliary obstruction.
22	14			3.5	3.9		
23	15			28	9		
Obstruction relieved.							
24	16	145	147	13	5.5		
25	17	151	151	4.5	2.1		
26	18	119	120	Tr.	1.3		
27	19	86	128	0	0		
28	20	72	112	0	0		
29	21	85	118	0	0	6½	
30	22	91	127	0	0		
							Interval of 10 days. No pigment determinations.
40	32	78	106	0	0		
41	33	90	120	0	0	6½	
42	34	86	120	0	0		
43	35	79	118	0	0		

TABLE III—*Concluded.*

Period after Operation.	Period after Infection of the biliary tract.	Amount of bile.	Total urobilin in bile.	Total urobilin in urine.	Total bilirubin in urine.	Weight.	Remarks.
days	*days*	*cc.*	*mg.*	*mg.*	*mg.*	*kg.*	
Total biliary obstruction.							Infection had endured over 5 wks. Gall bladder bladder no longer a normal organ, for autopsy, 51 days after intubation and 43 days after infection of the biliary tract, disclosed a greatly thickened gall bladder with pale green glazed contents. No evidence of concentration of the contents.
44	36			2+	5.3		
45	37			2+	13.9		
Obstruction relieved.							
46	38	138	156	3.5	7.6		
47	39	136	143	2.0	3.0		
48	40	108	130	1+ Tr.	1.5		
49	41	76	121	0	1.2		
50	42	81	129	0	0		
51	43	79	118	0	0	6¾	

TABLE IV.

Pronounced Urobilinuria Occurring in an Animal with Gall Bladder Connections Undisturbed and an Infected Biliary Tract. The Pigment Appeared after Injury by Chloroform and Again Later When Biliary Obstruction Was Produced.

Dog 14.

Period after Operation.	Period after Infection of the biliary tract.	Amount of bile.	Total urobilin in bile.	Total urobilin in urine.	Total bilirubin in urine.	Weight.	Remarks.
days	*days*	*cc.*	*mg.*	*mg.*	*mg.*	*kg.*	
Altercursive intubation. Gall bladder undisturbed. 7 days later.							The findings in this experiment are not charted.
7		120	0	0	0	11	
8		125	0	None voided.			
9		115	0	0	0		
Biliary tract deliberately infected.							The infective material, a bouillon suspension of dog fecal particles, was employed on the same day to infect Dogs 15, 16, 17, and 18.
10	1	126	23	0	0		
11	2	146	84	0	0		
12	3	137	130	0	0	10½	
Chloroform anesthesia. 70 cc.							Liver injury experiment performed prior to the induction of biliary obstruction.
13	4	43	55	Tr.	Tr.		
14	5	31	22	29.1	2.8		
15	6	22	28	36.4	4.5		
16	7	46	70	24.5	6.4		
17	8	74	70	6.5	2.4		
18	9	101	105	3.2	1.6		
19	10	130	108	Tr.	0		
20	11	122	120	0	0		
21	12	130	100	0	0	10¾	
22	13	118	135	0	0		
Total biliary obstruction.							Infection of the biliary tract had endured but 13 days. Pronounced urobilinuria followed total biliary obstruction.
23	14			9	2.1		
24	15			31	7.9		
Obstruction relieved.							
25	16	210	170	17.5	11.2		
26	17	180	145	5.8	3.1		
27	18	142	140	3.5	1.7		
28	19	116	105	2.0	Tr.		
29	20	134	115	0	0		Autopsy, 23 days after infection of the biliary tract and 32 days after intubation showed the gall bladder normal in appearance, its contents dark and highly concentrated.
30	21	121	136	0	0		
31	22	126	116	0	0		
32	23	135	102	0	0	10½	

TABLE V.

Pronounced Urobilinuria Occurring in an Animal with Gall Bladder Connections Undisturbed and an Infected Biliary Tract. The Pigment Appeared in the Urine When Liver Injury Had Been Produced with Chloroform.

Dog 13.

Period after Operation.	Period after Infection of the biliary tract.	Amount of bile.	Total urobilin in bile.	Total urobilin in urine.	Total bilirubin in urine.	Weight.	Remarks.
days	*days*	*cc.*	*mg.*	*mg.*	*mg.*	*kg.*	
Intubation. Gall bladder undisturbed. 4 days later.							The findings in this experiment are not charted.
4	St.	70	Tr.	0	0	7½	
5	"	72	Tr. to 0				
6	"	68	0	0	0		
Biliary tract infected.							For infection of the biliary tract the same material was employed as in Dogs 5, 8, 10, and 11.
7	1	76	36	0	0		
8	2	68	88	0	0		
9	3	72	95	0	0	7¾	
Chloroform anesthesia. 40 min. 65 cc.							Liver injury by chloroform anesthesia only. No biliary obstruction experiments.
10	4	41	26	6.8	1.5		
11	5	22	14	19	4.2		
12	6	12	7.5	24.3	7.4		
13	7	39	65	3.0	6.0		
14	8	48	78	2+	2.2		Early autopsy. Infection of the biliary tract has endured only 11 days. Gall bladder perfectly normal in appearance. Contents dark and highly concentrated.
15	9	77	90	Tr.	Tr.+	7¼	
16	10	72	88	0	0		
17	11	69	99	0	0	7½	

TABLES VI AND VII.

Trifling Urobilinuria Occurring in Animals with Obstruction of the Cystic Duct and an Injected Biliary Tract. The Pigment Appeared in the Urine When Biliary Obstruction Was Produced and Again Later When the Liver Was Injured with Chloroform.

TABLE VI.

Dog 7.

Period after Operation.	Period after Infection of the biliary tract.	Amount of bile.	Total urobilin in bile.	Total urobilin in urine.	Total bilirubin in urine.	Weight.	Remarks.
days	*days*	*cc.*	*mg.*	*mg.*	*mg.*	*kg.*	
Altercursive intubation. Gall bladder excluded. 81 days later.							These findings are not charted.
81	St.	134	0	0	0	10½	
82	"	128	0	0	0		
83	"	132	0	0	0		
Biliary tract deliberately infected.							The biliary tract was infected by injection into it, previously sterile, bile which had been inoculated with a bouillon suspension of dog fecal particles.
84	1	122	6	Tr.	Tr.		
85	2	138	84	0	0		
86	3	127	153	0	0		
87	4	132	135	0	0		
Total biliary obstruction.							Though infection of the biliary tract had endured but 4 to 8 days only traces of urobilin were found in the urine following biliary obstruction.
88	5			1.0	2.0	10	
89	6			2.0	9.5		
90	7			Tr.	13.7		
Obstruction relieved.							Biliary obstruction induced prior to the liver injury experiment.
91	8	160	155	1.0	0		
92	9	162	130	0	0		
93	10	139	160	0	0		
94	11	110	160	0	0		
Chloroform anesthesia. 45 min.							
95	12	59	140	2	4.65	10¼	
96	13	48	54	Tr.	2.57		Again only traces of urobilin in the urine.
97	14	19	13	2+	11.85		
98	15	58	66	1.0	4.25		
99	16	74	83	Tr.	3.92		
100	17	108	107	"	Tr.		Autopsy 20 days after infection of the biliary tract and 103 days after intubation showed the gall bladder still excluded from the biliary channels.
101	18	113	141	0	0		
102	19	134	132	0	0		
103	20	127	144	0	0	10¼	

TABLE VII.

Dog 3.

Period after Operation.	Period after Infection of the biliary tract.	Amount of bile.	Total urobilin in bile.	Total urobilin in urine.	Total bilirubin in urine.	Weight.	Remarks.
days	*days*	*cc.*	*mg.*	*mg.*	*mg.*	*kg.*	
Altercursive intubation. Gall bladder excluded. 87 days later bile became infected.							The findings are not charted.
87	1					16½	The bile became spontaneously infected, turned orange-brown, and contained urobilin. This bile was used to infect the biliary tracts of Dogs 5, 8, 11, and 13, all on 1 day, and that of Dog 10 6 days later.
105 days after intubation.							
105	19	200	167	0	0		
106	20	205	140	0	0		
107	21	178	156	0	0	16½	
Total biliary obstruction.							Biliary obstruction induced prior to the liver injury experiment.
108	22			2.0	8.7		
109	23			3.0	27.6		
Obstruction relieved.							
110	24	235	190	2.0	18.4		
111	25	175	195	Tr.	4.6		
112	26	190	166	"	2.2		
113	27	175	158	"	Tr.		
114	28	210	173	0	0		
115	29	200	142	0	0		
116	30	180	168	None voided.			
117	31	170	138	0	0	17	
Chloroform anesthesia.							
118	32	110	92	0	3.7		
119	33	62	38	Tr.	9.4		
120	34	22	30	2	18.5	16½	
121	35	68	62	2	6.8		
122	36	102	134	2	3.3		
123	37	175	160	Tr.	2+		Autopsy 40 days after infection of the biliary tract and 126 days after intubation showed the gall bladder still excluded from the biliary channels.
124	38	210	140	0	F.tr.		
125	39	182	156	0	0	16½	
126	40	201	168	0	0		

TABLES VIII AND IX.

Trifling Urobilinuria Occurring in Animals with Obstruction of the Cystic Duct and an Infected Biliary Tract. The Pigment Appeared in the Urine after Liver Injury by Chloroform and Again Later When Biliary Obstruction Was Produced.

TABLE VIII.

Dog 15.

Period after Operation.	Period after Infection of the biliary tract.	Amount of bile.	Total urobilin in bile.	Total urobilin in urine.	Total bilirubin in urine.	Weight.	Remarks.
days	*days*	*cc.*	*mg.*	*mg.*	*mg.*	*kg.*	
Altercursive intubation. Gall bladder excluded. 5 days later.							The findings in this experiment are not charted.
5	St.	103	0	F. tr.	0	9½	
6	"	111	0	0	0		
7	"	84	0	0	0		
Biliary tract deliberately infected.							The same material was used on the same day to infect the biliary tracts of Dogs 14, 16, 17, and 18.
8	1	89	14	0	0		
9	2	92	44	0	0		
10	3	88	61	0	0		
Chloroform anesthesia. 30 min.							
11	4	61	52	None voided.		8¾	
12	5	26	33	Tr.	1.5		Though infection of the biliary tract had endured but 4 to 8 days, only traces of urobilin were found in the urine following liver injury.
13	6	12	12.5	2.0	6.7		
14	7	38	56	2.0	11.1		
15	8	44	66	Tr.	3.5	8½	Liver injury experiment performed prior to the induction of biliary obstruction.
16	9	88	83	0	2.1	8	
17	10	80	70	0	1+		
18	11	79	79	0	0		
19	12	94	68	None voided.			
20	13	84	81	0	0	8¾	
Total biliary obstruction.							
21	14			Tr.	2.6		
22	15			"	11.5		
Obstruction relieved.							
23	16	142	114	2.0	14.6	8½	
24	17	154	121	2.0	9.2		
25	18	90	88	2.0	5.4		
26	19	81	94	Tr.	2.2		
27	20	97	79	0	2.1		
28	21	86	84	None voided.			Autopsy 24 days after infection of the biliary tract and 31 days after intubation showed the gall bladder still excluded from the biliary channels.
29	22	85	80	0	0		
30	23	91	95	0	0		
31	24	79	78	0	0	8½	

TABLE IX.

Dog 16.

Period after Operation.	Period after Infection of the biliary tract.	Amount of bile.	Total urobilin in bile.	Total urobilin in urine.	Total bilirubin in urine.	Weight.	Remarks.
days	*days*	*cc.*	*mg.*	*mg.*	*mg.*	*kg.*	
Altercursive intubation. Gall bladder excluded. 5 days later.							The findings in this experiment are not charted.
5	St.	84	0	0	0	11¾	
6	"	81	0	0	0		
7	"	77	0	0	0		
Biliary tract infected deliberately.							The same infective material was used on the same day to infect the biliary tracts of Dogs 14, 15, 17, and 18.
8	1	82	51	None voided.			
9	2	101	96	0	0		
10	3	120	102	0	0		
Chloroform anesthesia. 35 min.							
11	4	66	41	Tr.	3.1	11½	Though infection of the biliary tract had endured but 4 to 8 days only traces of urobilin were found in the urine following liver injury.
12	5	24	22.5	"	8.4		
13	6	18	20	2	7.9		
14	7	34	52	2	4.5		Liver injury experiment performed prior to the induction of biliary obstruction.
15	8	76	94	Tr.	1.8	11	
16	9	84	120	0	0		
17	10	72	111	0	0		
18	11	69	118	0	0		
19	12	78	98	0	0	10½	
Total biliary obstruction.							
20	13			Tr.	4.7		
21	14			2.0	24.4		
Obstruction relieved.							
22	15	181	167	Tr.	26.4		
23	16	132	174	2.0	14.3		
24	17	82	102	Tr.	6.1		
25	18	88	91	None voided.			
26	19	79	106	Tr.	1+		Autopsy 22 days after infection of the biliary tract and 29 days after intubation showed the gall bladder still excluded from the biliary channels.
27	20	91	115	0	0		
28	21	86	89	0	0		
29	22	82	99	0	0	10½	

STUDIES ON THE EFFECT OF CERTAIN TOXIC SUBSTANCES IN BACTERIAL CULTURES ON THE MOVEMENT OF THE INTESTINES.

I. The Effect of Soluble Toxic Substances of Young Cultures of Bacillus paratyphosus B.*

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HISTORICAL.

Since the classical studies of Gärtner (1) considerable work has been done to demonstrate the occurrence of toxins in cultures of organisms belonging to the paratyphoid-enteritidis group. The literature on this subject has been reviewed by Ecker (2) and by Branham (3). It has been shown by Ecker (2) and others that rabbits, upon intravenous injection of 2 to 5 cc. of a Berkefeld filtrate of young cultures of these organisms became weak, prostrated, and dyspneic in from 1 to 2 hours. The animals often exhibited a profuse diarrhea, while control animals receiving broth alone showed no symptoms. Smith and TenBroeck (4) in a study of the fowl typhoid bacillus observed that a dog receiving 1 cc. per kilo of a filtrate of a culture of their organism showed a diarrhea, with the passage of blood and mucus, and persistent vomiting. At the end of 24 hours there was a prolapse of the intestine and the animal was killed. On necropsy it was found that at least 2 feet of the small intestine had been invaginated through the ileocecal valve and had moved down through the large intestine and out through the anus. The authors believe that peristalsis is markedly stimulated by the filtrate. Geiger, Davis, and Benson (5) fed rabbits by stomach tube with food contaminated with organisms of the paratyphoid-enteritidis group and its filtrates, boiled and unboiled. Thirty rabbits were used for each experiment of each strain of organism. They observed symptoms of diarrhea once in a series of experiments with *B. paratyphosus* C, twice in the *B. paratyphosus* B series, and eight times in the *B. paratyphosus* A series. Of animals receiving fresh filtrates, diarrhea was noted

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in only two instances and these in the *B. paratyphosus* A series. We have not observed diarrhea in rabbits following feeding by stomach tube of 25 cc. of toxic filtrate of *B. paratyphosus* B, or *B. suipestifer*. Once a slight diarrhea was noted in a cat fed 25 cc. of a filtrate of *B. suipestifer*. We agree with Geiger, Davis, and Benson that rabbits have considerable tolerance to bacteria of the paratyphoid-enteritidis group and their filtered products, when placed directly into the gastrointestinal canal. However, upon intravenous injection we found that with certain strains of *B. paratyphosus* A and B, *B. suipestifer*, *B. enteritidis*, and *B. paratyphosus* C, diarrhea often occurs.

In the present study an attempt was made to observe directly the action of the toxic filtrates on intestinal movements because the existing data appeared unsatisfactory.

Friedberger and Kumagai (6) attempted to study the endotoxins of *B. typhosus*, *B. dysenteriæ* (Shiga), and the vibrio comma, on the isolated intestine by the method of Magnus, but failed to obtain a reaction. The intestines of both normal and immune animals were used. The only reaction they observed was when an anaphylatoxin was prepared. These authors claim that the homologous serum employed in the preparation of the anaphylatoxin did not affect the isolated segment.

It has been pointed out by de Graaff (7) that *B. paratyphosus* may produce para-oxyphenylethylamine and β indoleethylamine. Both of these compounds are known to be physiologically active. According to a recent study of Nakamura (8) para-oxyphenylethylamine in small doses tends to decrease the tone and movements of rabbit's intestines, while larger doses, following a distinct drop, produce a sudden rise of tone. Laidlaw (9) states that indoleethylamine has a direct stimulant action on plain muscle. Its physiologic action is intermediate between that of the sympathomimetic monamines, such as *p*-hydroxy-phenylethylamine, and the diamines, like imidazoleethylamine. With the Magnus method, Ecker and Siaulis[1] failed to obtain an effect on the isolated segment, indicating that we must be dealing with different substances. Furthermore, Branham reports that filtrates of *B. enteritidis* were found to contain a maximum of 0.000625 gm. of histamine in 200 cc. and that the usual amount injected was too small to have any but the most transient effect on the rabbit. Upon inoculation of material containing this trace of histamine, enough to equal the 2 cc. of the filtrate which they gave no effects were produced. It must be pointed out that the peptone may have contained this trace of histamine as no study of the broth alone was made by the author. Blankenhorn, Harmon, and Hanzlik (10) in a study of an outbreak of ptomain poisoning from "creamed codfish" reported that extracts of the "creamed" fish gave practically the same physiologic reactions as the same brand

[1] To be published.

of salted codfish which was previously allowed to putrefy and then was prepared in the same manner as the food served to the patients. The physiologic effects consisted of a marked stimulation of intestinal and uterine peristalsis with isolated surviving organs, and a fall of blood pressure with gradual recovery. The purified active extract of the creamed putrefied salted codfish contained some physiologically active base, whose chemical reactions resemble those of the group of diamines. The only organism found was *B. coli communior*. In these experiments the food was allowed to decay and it is therefore likely that active amino bases may occur, accounting for their results.

In our experiments young culture filtrates (less than 24 hours) have been employed and all the experiments have been carried out with the organ *in situ*, in the living animal.

Method.

Several strains of *B. paratyphosus* B were employed. Nos. 180 and 185 gave the most satisfactory toxins and No. 180 was particularly active in the production of a diarrhea. No. 185 caused a more violent respiratory reaction. The organisms were grown for 15 to 24 hours at 37°C., on 2 per cent Witte peptone veal infusion broth with a pH of ± 7. Witte peptone gave the most satisfactory results. The cultures were then filtered through a Berkefeld N candle and were always used immediately after filtration. In the early experiments we have grown Nos. 180 and 185 together and have also mixed equal volumes of the two filtrates to obtain maximal effects, but the filtrate of either strain will cause the same marked reactions, considering variability of toxicity of the filtrate and susceptibility of the animal. The sterile broth was used as a control. Paralleling these experiments normal rabbits (2 to 3 kilos) were given intravenous injections of the filtrates in order to ascertain their activity. The animals destined for operation were anesthetized and rendered immobile by a large dose of urethane (2 gm. per kilo) administered by stomach tube. The animals were given no food for at least 24 hours prior to the experiment.

The first method used for the study of the movements of the small intestines *in situ* in the living animal was the "pouch method" of Sollmann (11). An incision, about 15 cm. in length, was made through the skin of the abdomen in the median line. Following blunt dissection of the skin from its underlying fascia, the skin was suspended from an oval ring measuring 15 by 17 cm. The ring was then raised with a suitable V-shaped support to form a water-tight pouch. The sac was filled with warm Locke's solution,[2] carefully kept at constant temperature

[2] The solution used by us contains, per liter H_2O: NaCl 9.0 gm.; KCl 0.42 gm.; $CaCl_2 2H_2O$, 0.24 gm.; $NaHCO_3$ 0.3 gm.; glucose 1 gm. pH = 7.8.

(38.5–39°C.) by inflowing warm Locke's solution. The excess fluid flowed over the border of the ring. An incision (± 3 cm.) was then made through the wall of the abdomen and a loop of the small intestine with a fairly large mesentery was brought out. The wound was sutured to prevent the escape of other loops. The selected segment was then horizontally hooked up between two fixed pins and by means of a serrefine or pin, thread and lever the pendulum movements were recorded in the usual way. The lever registrations were always supplemented by direct inspection. Following preparation of the animal, from ½ to 1 hour was allowed to elapse before any registration was made and the experiments were begun when the pendulum movements were regular and free from spontaneous changes. At times 2 hours were necessary to await the disappearance of spontaneous changes and an occasional animal was encountered in which the irregularities of contraction were such as to make experimentation impossible.

In the study of the propulsive efficiency of the normal segments *in situ*, and in the living animal, we employed the Sollmann and Rademaekers (12) modification of an arrangement described by Max Baur (13). The technique with this arrangement is more complicated and for its description the reader is referred to the original publication. By this method we were able simultaneously to study and record not only the longitudinal and circular muscle contractions but also the propulsive efficiency of the segment for a given time.

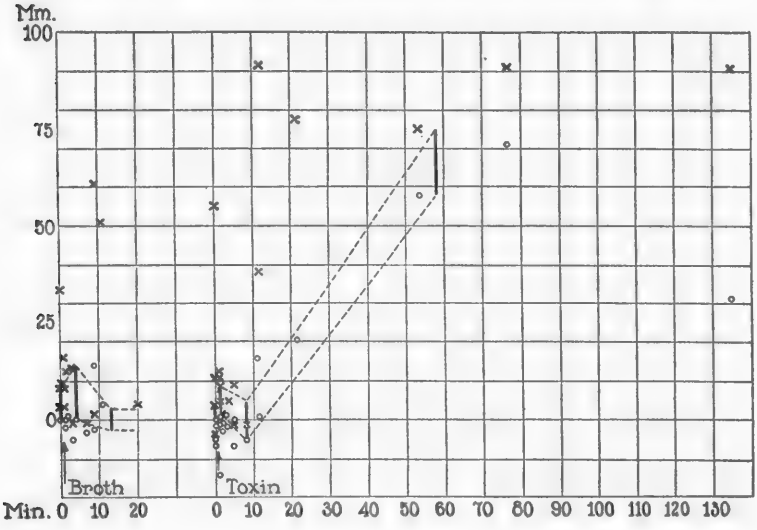
In charting the results of these experiments we have measured in mm. the "diastolic" and "systolic" tones at points of definite changes. These were plotted on the ordinates and the time in minutes on the abscissæ. The median was then taken to indicate the changes.

The Effect of Intravenous Injection of Filtrates on the Pendulum Movements of the Upper Intestine.

In a series of five experiments with parallel controls the rabbits were first injected intravenously with sterile broth (2 to 5 cc.) to study the effect of the broth. A slight rise of tone occurred immediately after intravenous injection of the broth. A similar reaction was seen when physiologic salt solution was injected or a simple puncture of the vein was made. Shaving of the ear may induce identical results. This effect never lasted more than 3 or 4 minutes following the injection of the broth, when the contractions returned to normal or slightly below. Text-fig. 1 shows that a systolic rise of 6 mm. occurred in these animals (median of five experiments) which at the end of 4 minutes disappeared again. We have always allowed from 1 to 3 hours to elapse after the injection of the broth and before the toxic filtrate was administered in order to ascertain whether or not

the broth itself may produce a late rise in tone. This did not occur in any case, thus confirming the clinical observations. A dose of 10 cc. of sterile broth in the clinical experiments failed to produce any symptoms in the animals.

Text-fig. 2 shows the effect of the filtrate on the tone of the longitudinal muscles. It is noted that the same immediate rise of sys-



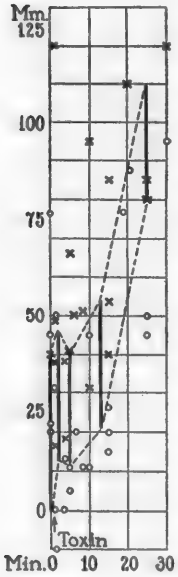
TEXT-FIG. 1. TEXT-FIG. 2.

TEXT-FIG. 1. The effect of intravenous injection of sterile broth on the tone of the longitudinal muscles of the upper intestines of the rabbit. (Median of five experiments.)

TEXT-FIG. 2. The effect of the toxic filtrate on the longitudinal muscles of the upper intestines of the rabbit. Preceding the marked rise of tone an immediate slight rise of the tone due to the injection is observed. (Median of five experiments.)

tolic tone occurred. Here the drop came at the end of the 2nd minute and progressed in a further drop to a diastolic of −5 and a systolic of +5, by the end of the 8th minute. From here on the phenomena changed to a second, slow but vastly powerful, rise of tone reaching its maximum at the end of 58 minutes. The diastolic

rise reached 58 mm. and the systolic 70 mm. The reaction was persistent in character as demonstrated in Text-fig. 4. In Text-fig. 2 the reaction was late in two of the animals. One of these (slower one) received a phenol-containing toxic filtrate. We do not know whether this accounts for its late effect or not.



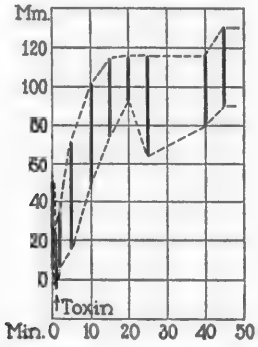
TEXT-FIG. 3. Showing the increase of tone of the longitudinal muscles of the upper intestines of the rabbit due to the toxic filtrate injection. No previous injection of broth was given. (Median of five experiments.)

In a subsequent series of five animals no preliminary broth injections were given. The animals received the usual amount of the toxic filtrates in the ear vein. Prior to the experiment, intact control animals received the same filtrates to determine their toxicity. The reaction in these animals was acute and diarrhea was always present. Text-fig. 3 illustrates the results obtained. Again the median of five experiments was plotted. The normal amplitude here was large and following the same immediate rise (broth effect) the tone gradually rose reaching its maximum at the end of 25 minutes. The amplitude at this point was shorter; *i.e.*, the spasm was such that the intestine relaxed but little between peristaltic waves. The diastolic rise was from 11 mm. to 80 mm. and the systolic from 42 mm. to 110 mm. A definite rise occurred at the end of 13 minutes (diastolic from 11 mm. to 21 mm. and systolic from 42 mm. to 54 mm.). An incubation period of a minimum of at least 10 minutes passed before the rise was noted. This period varies in different animals and with different filtrates.

Text-fig. 4 shows the persistency of the rise. Fluctuations of course occur but the tone remains high and in this case a still higher point was reached at the end of 50 minutes. The toxic filtrate employed in this experiment contained phenol (0.4 per cent), which accounts for the immediate brief drop of tone following injection of the filtrate.

We have further observed instances of a persistent high tone lasting for 2 hours, following intoxication.

It was found that magnesium chloride or sulfate, 5 to 10 cc. of isotonic solutions ($MgCl_2$ 2.1 per cent and $MgSO_4$ 3.25 per cent) added directly to the bath, relaxed the toxin spasm and quieted the excessive peristalsis. Text-fig. 5 (mean of four experiments) clearly demonstrates the effect of the salts. Within 14 minutes after the addition of the salt the tone dropped and with this drop the amplitude was also decreased. The diastolic tone of the intestine was 50 mm., the systolic 110 mm., and the amplitude therefore 60 mm. At the end of 14 minutes the diastolic tone was 13 mm., the systolic 22 mm., and the amplitude 9 mm.



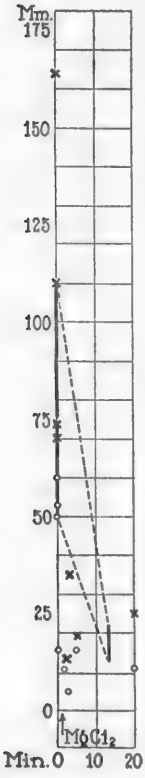
TEXT-FIG. 4. Showing the persistency of a high tone of the longitudinal muscles of the upper intestines of the rabbit following the administration of the toxic filtrate.

In Tracings 1, *A*, *B*, and *C* (longitudinal muscle movements) the original contractions were small. The effects of broth and toxin injection are clearly demonstrated. Tracing 1, *B* shows the rise of tone and Tracing 1, *C* shows the maximal effects obtained. Evidence of peristalsis is here seen. Peristalsis occurred during the rise.

The Effect of the Filtrate on Peristalsis and Propulsion in the Small Intestine.

The preceding experiments have definitely shown that the toxic filtrates stimulate the longitudinal muscle of rabbits' intestine. Since it is not known, however, that the longitudinal muscle always reflects

the activity of the circular muscles, on which peristalsis depends, another series of experiments was made in which the peristaltic propulsion was measured by the propulsion arrangement. With this method, an interval of usually 2 to 3 hours elapses before good peristalsis becomes established. Dr. Sollmann thinks that this is due to depression of the intestinal muscles by the large dose of urethane that is used to secure complete immobilization of the animal. This is gradually washed out by the peritoneal lavage, so that the intestines recover their activity. In the interval, the intestinal tone is low. Sollmann and Rademaekers observed that 6 to 8 cm. of water failed to provoke peristalsis under these circumstances. It is therefore of particular interest that the toxic filtrate injected during this period almost invariably started the peristaltic waves. Again, in these experiments the effect of sterile broth injections on the propulsive efficiency of the segment was carefully studied, and compared with the effect of a toxic filtrate injection. In other experiments we have allowed sufficient time to elapse following administration of the broth to overcome the temporary inhibition of peristalsis and when the waves became regular the filtrate was injected. Lastly, the toxic filtrate was injected without any previous broth inoculations. All these experiments were controlled with unoperated animals, which received the same amount of the filtrates.



TEXT-FIG. 5. Showing the relaxing effect of isotonic magnesium chloride. (Median of four experiments.)

In Experiment III (output arrangement), the rabbit, 1950 gm., received 4.5 gm. urethane by stomach tube, 3 cc. broth by ear vein, and later 3 cc. of an 18 hour toxic culture filtrate of *B. paratyphosus* B Nos. 180 and 185. The quantity of fluid propelled by the peristalsis of the intestinal segment was increased to about three and one-half times by the toxin. 84 dipping buckets were discharged in 108 minutes following the injection of the broth; but 298 buckets in the same time (109 minutes) following the injection of the filtrate.

Tracings A, B, and C of Fig. 2 demonstrate the reaction.

In a second experiment, No. IV, under the same conditions (rabbit 2600 gm. 5 gm. of urethane, 5 cc. of a 15½ hour culture filtrate of *B. paratyphosus* B Nos 180 and 185) the output was increased to four times, *i.e.* from 25 buckets in 100 minutes following broth administration to 126 buckets in 120 minutes, thus about 1 bucket per minute. In this case the waves occurred before the sterile broth was injected. Two control animals showed marked defecation from ½ to 1 hour following injection of the toxin, and at night soft stools were evacuated by these animals. They exhibited the same typical picture of intoxication.

In a third experiment of this kind (rabbit 1600 gm., urethane 3.0 gm., filtrate of 19 hour culture of *B. paratyphosus* B Nos. 180 and 185, 4 cc. intravenously), the output started 26 minutes after the injection of the toxic filtrate. No preliminary broth injection was given. In a total of 127 minutes the segment dumped 27 buckets. A control rabbit of the same weight evacuated soft feces 55 minutes after the injection of the toxic filtrate, and exhibited all the usual symptoms. 3 hours after the injection this control animal evacuated markedly. In Experiment VI with the same arrangement (rabbit 1350 gm., 3.0 gm. of urethane, filtrate of 18 hour culture of *B. paratyphosus* B Nos. 180 and 185), the animal received two injections, each time 3 cc. sterile broth, and sufficient time was allowed to elapse so as to obtain a normal peristalsis. The second broth injection was given 1 hour and 15 minutes after the first. Following the second injection the output was, in 56 minutes, 62 buckets, and following the toxin injection it was, in 88 minutes, 93 buckets, thus not so marked an increase as in the previous experiments. A control rabbit weighing 1870 gm. received the same amount of the toxic filtrate and showed a marked reaction with typical diarrhea in 1 hour after the injection of the toxic filtrate. The interesting part of this experiment is the fact that the waves were spastic, travelling slowly, following the injection of the toxic filtrate, and the output therefore not greatly increased.

A mild effect was obtained with the filtrate of a strain of an organism of the paratyphoid group freshly isolated from a food poisoning outbreak.

Incidentally, it was also observed that the rabbits under urethane did not exhibit as much diarrhea as the control animals without anesthetic. In one instance a most profuse diarrhea occurred in one animal after it came out of the anesthesia. In other words, the urethane tends to diminish the diarrhea.

SUMMARY.

Following intravenous injection, filtrates of young cultures of *B. paratyphosus* B often produce marked diarrhea in rabbits. A study was made of the effect of these toxic filtrates on the motility

of the small intestines of the rabbit. The observations were made on a segment of the small intestines *in situ*, and in the living animal. It was found that an immediate slight rise of tone of the longitudinal muscles occurred following intravenous injection of sterile broth. The same rise was noted after the injection of the toxic filtrate; but with these it was followed later (10 minutes elapsing at least) by a very strong but gradual rise of the diastolic and systolic tone, *i.e.*, by spasmodic contraction of the intestinal muscle, which persisted at times for as long as 2 hours. In order to record simultaneously the effect on the longitudinal and circular muscles, and the propulsive efficiency of the segment the Sollmann and Rademaekers modification of Baur's technique was employed. This arrangement showed that the stimulation of the longitudinal muscles is accompanied by a similarly strong stimulation of the circular muscles, by peristalsis, and therefore by a greatly increased propulsion of intestinal contents which was sufficient to overcome the inhibition that usually occurs after preparation of the animal. With this arrangement an instance of peristaltic spasm was also noted. Broth alone failed to produce the phenomenon. Isotonic magnesium chloride or sulfate added to the bath relaxed the muscles again. Animals under deep urethane anesthesia did not show the diarrhea occurring in the intact controls, but sometimes exhibited it after the effect of the anesthetic had disappeared. So far no effects have been observed on the isolated strip (Magnus method), and further studies are being made to localize the effect, to neutralize it with a specific antiserum, and to observe the effect of filtrates of other members of the bacterial group including the dysentery bacilli.

We desire to acknowledge our gratitude to Dr. T. Sollmann for his continuous interest in the progress of the work.

BIBLIOGRAPHY.

1. Gärtner, A., *Cor.-Bl. allg. ärztl. Ver. Thüringen*, 1888.
2. Ecker, E. E., *J. Infect. Dis.*, 1917, xxi, 541. Ecker, E. E., and Richardson, M. L., *J. Infect. Dis.*, 1925, xxxvii, 538. Ecker, E. E., and Megrail, E., *J. Infect. Dis.*, 1925, xxxvii, 546.
3. Branham, S. E., *J. Infect. Dis.*, 1925, xxxvii, 291.

4. Smith, T., and TenBroeck, C., *J. Med. Research*, 1914–15, xxxi, 523.
5. Geiger, J. C., Davis, E., and Benson, H., *Am. J. Pub. Health*, 1924, xiv, 578.
6. Friedberger, E., and Kumagai, T., *Centr. Bakt., 1. Abt., Ref.*, 1912, liv, suppl., 39.
7. de Graaff, W. C., De biochemische Eigenschappen der Paratyphusbacillen, Leiden, 1919, 72.
8. Nakamura, M., *Tohoku J. Exp. Med.*, 1925, vi, 367.
9. Laidlaw, P. P., *Biochem. J.*, 1912, vi, 141.
10. Blankenhorn, M. A., Harmon, G. E., and Hanzlik, P. J., *Arch. Int. Med.*, 1916, xvii, 140.
11. Sollmann, T., *Am. J. Physiol.*, 1922, lxiii, 395.
12. Sollmann, T., and Rademaekers, A., *Arch. internat. pharmacod. et thérap.*, 1925, xxxi, 39.
13. Baur, M., *Arch. exp. Path. u. Pharmakol.*, 1923, c, 95.

EXPLANATION OF PLATE 28.

FIG. 1 (tracings). The effect of the toxic filtrates on the pendulum movements of the upper intestines of the rabbit. (*A*) Demonstrating the immediate rise of tone and amplitude obtained after the injection of the broth and filtrate. (*B*) Demonstrating a marked increase of tone and amplitude due to the injection of the toxic filtrate. (*C*) Demonstrating the maximal effect produced. Peristaltic waves were observed.

FIG. 2 (tracings). The effect of the toxic filtrates on intestinal movements. Pendulum, circular, and propulsion. (*A*) Demonstrating the effect of sterile broth. (*B*) The effect of the toxic filtrate. (*C*) Maximal reaction obtained. The output is almost continuous.

Uppermost tracing: longitudinal contractions.
Second tracing: pressure curve.
Third tracing: propulsion.
Fourth tracing: time in minutes.

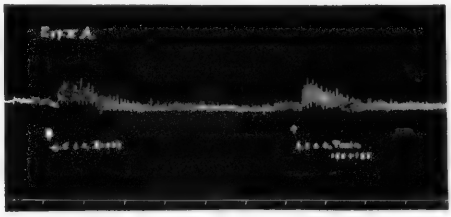


FIG. 1, *A*.

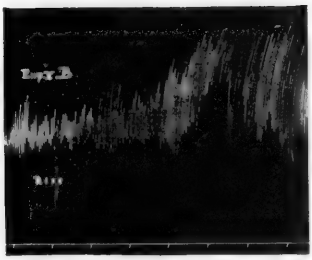


FIG. 1, *B*.

FIG. 1, *C*.

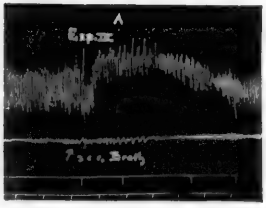


FIG. 2, *A*.

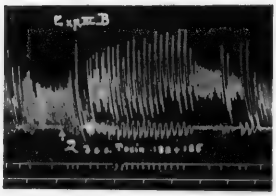


FIG. 2, *B*.

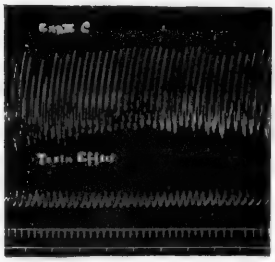


FIG. 2, *C*.

(Ecker and Rademaekers: Toxic substances in bacterial cultures. I.)

UNIV. OF ILL.

THE RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNITY.

II. The Complement Titer after Blockade and the Physiological Regeneration of the Reticulo-Endothelial System as Measured by Reduction Tests.

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INTRODUCTION.

Study of the reticulo-endothelial system in its relation to the production of antibodies has so far not included observations on the titer of complement in animals in which this system has been blocked by intravenous injections of some colloidal substance, except for a brief statement by Siegmund (1) who mentions that the production of complement in the blocked animal is not changed. While the adsorption of enzymes by a number of substances of fine dispersion is a well known fact (2) and while, furthermore, it has been reported that complement is adsorbed by many different substances of colloidal nature *in vitro* (3), such as cellular suspensions (4), certain precipitates (4), gelatin (4), kaolin (4, 5), dyestuffs (6), starch (7), and charcoal (4), etc., the action of similar substances on the titer of complement *in vivo* seems to have been studied less extensively. Loewit (8) states that after the injection of colloids (agar) a decrease of complement may be observed.

In the course of experiments undertaken to determine, at various intervals, the complement titer in guinea pigs which had received one blocking injection of India ink, the opportunity also arose of studying by physiological chemical methods the progress of such a blockade at the same intervals. This work seemed to be of particular interest, since in a previous paper (9) on the production of diphtheria antitoxin in the blocked animal, it was found that an initial phase, during which

no antitoxin was present, was followed rather quickly by the appearance of quantities of the antibody corresponding to the titer of the controls.

EXPERIMENTAL WORK.

Guinea pigs were chosen as test animals and Higgins' insoluble India ink as the blocking substance. Eighteen guinea pigs of approximately 300 to 350 gm. weight were injected intravenously[1] with 1.5 cc. of a 1:5 ink dilution and the complement titer of the blood was tested after different intervals of time. The normal complement titer of nine of the eighteen animals was tested before the experiment was begun; the blood in these cases was obtained by heart puncture; for the final test the animals were bled to death. Duplicate guinea pigs were always run for each time interval. The disadvantage of testing different guinea

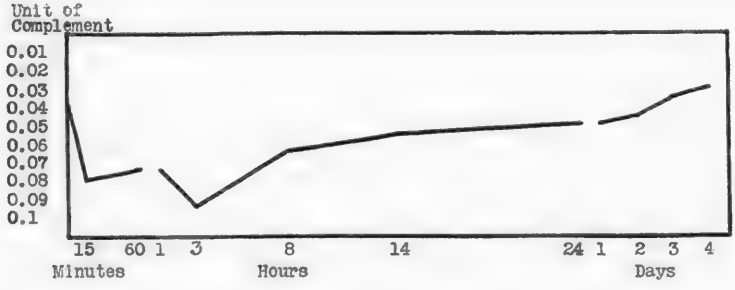

CHART 1. Complement unit after intravenous injection of India ink.

pigs at different intervals instead of following the course of the complement titer in the same animal is fully realized, but this disadvantage was thought to be more than compensated for by the avoidance of repeated heart punctures at short intervals and of the danger of causing anemia and subsequent artificial changes in the complement titer, by frequent bleeding. Simultaneously two controls, which received an injection of physiological salt solution instead of ink, were run. The complement titer of the serum in one of these animals was tested before the injection of the salt solution. Table I and Chart 1 give the results of these experiments.

It appears from Table I and Chart 1 that there occurs a marked drop of complement in the circulation, after an intravenous injection of ink, which sets in as early as 15 minutes after the injection but does not reach its maximum until about 3 hours later. From then on, the

[1] All injections were made into the superficial vein on the dorsal side of the calf of the left hind leg as described in detail in an earlier paper (9).

TABLE I.

The Effect of India Ink Injected Intravenously into Guinea Pigs on the Titer of Complement.

Guinea pig No.	Complement unit* before ink injection. Aug. 5, 1925.	Interval between ink injection and bleeding for titration of complement.	Complement unit after ink injection. Aug. 12, 1925.
1	0.04	4 days.	0.03
2			0.03
3	0.04	3 "	0.04
4			0.03
5	0.05	2 "	0.05
6			0.04
7	0.04	24 hrs.	0.05
8			0.05
9	0.03	14 "	0.06
10			0.05
11	0.04	8 "	0.06
12			0.07
13	0.05	3 "	0.09
14			0.1
15	0.04	1 hr.	0.07
16			0.08
17	0.04	15 min.	0.08
18			0.08
19†	0.04		0.04†
20†			0.05†

Guinea pig serum dilution (final dilution of complement 1:20): 0.1 cc.
Red sheep cells sensitized with 2 units of hemolytic amboceptor: 0.2 cc.
NaCl solution: 0.2 cc.
Incubated 20 minutes at 37°C.

* The complement unit is the smallest amount of guinea pig serum giving complete hemolysis under the conditions of the test.

† Controls: Received 1.5 cc. of a 0.85 per cent salt solution intravenously 4 hours before bleeding for titration of complement.

complement titer rises gradually again, attaining practically its normal level 24 hours after the injection. Complement titrations carried out 3 and 4 days, respectively, after the ink injection showed evidence of a slight increase in complement as compared with the normal level.

These results made it desirable to study the action of India ink on the complement titer of guinea pig serum *in vitro* under similar conditions. 3 cc. of guinea pig serum were mixed with 3 cc. of a 1:5 ink dilution and kept in the ice box. Simultaneously 3 cc. of the same guinea pig serum were mixed with 3 cc. of salt solution as a control.

TABLE II.

The Effect of India Ink on the Complement Titer of Guinea Pig Serum in Vitro.

Test.		Control.	
Guinea pig serum + India ink dilution 1:5.		Guinea pig serum + physiological salt solution.	
Time of contact.	Complement unit.	Time of contact.	Complement unit.
			cc.
5 min.	No hemolysis up to 0.14 cc.	5 min.	0.05
30 "	" " " " 0.14 "	30 "	0.05
1 hr.	" " " " 0.14 "	1 hr.	0.06
5 hrs.	" " " " 0.14 "	5 hrs.	0.07

Guinea pig serum dilution (final dilution of complement 1:20): 0.1 cc.
Red sheep cells sensitized with 2 units of hemolytic amboceptor: 0.2 cc.
NaCl solution: 0.2 cc.
Incubated 20 minutes at 37°C.

The complement unit is the smallest amount of guinea pig serum giving complete hemolysis under the conditions of the test.

After different intervals of time 0.5 cc. were removed from each mixture and diluted with salt solution in the proportion of 1:10, thus yielding a final 1:20 dilution of the complement. It proved to be impossible to eliminate the carbon particles of the ink in the original concentration either by filtration through paper or by centrifugalization, while in the final dilution carbon particles were almost completely thrown down by centrifugalization. After incubation, then, all tubes were centrifugalized and the absence or presence of hemolysis was determined in the supernatant fluid. Microscopical examination of the sediment showed the carbon particles lying apart from the erythro-

cytes, thus excluding the possibility that absence of hemolysis may have been due to changes in the cell membrane caused by the ink. The titrations of the complement titer gave the results contained in Table II.

It appears from Table II that complement was completely adsorbed to the ink in the amounts tested after 5 minutes contact and that after 5 hours contact this adsorption effect was apparently still present. The deterioration of the complement in the control test made it undesirable to prolong this experiment beyond the period of 5 hours.

The question arises: What causes the drop of complement in the animal after the intravenous injection of ink? Is it merely a phenomenon of adsorption of the complement in the circulation to the carbon or is it due to a temporarily impaired function of the reticulo-endothelial system, assuming that this is the site of complement formation? The almost immediate disappearance of the carbon particles from the blood stream[2] and the fact that the maximum of this drop is not reached until 3 hours after the injection would speak against a mere adsorption mechanism.

It seemed of interest to submit further experimental evidence in support of Aschoff's contention (10) that blocked organs, though macroscopically still quite black for a long time after the ink injection, are restored in a comparatively short time to their physiological integrity by regenerative processes of the reticulo-endothelial tissue. The histological examination could not be expected to furnish much information on this point because functionally the tissue might be again at the height of its activity before the cells showed morphological signs of regeneration. It was decided to adopt the rate of reduction of these organs as a criterion of their physiological activity. The indicators used for measuring the respiratory metabolism of the cells were first, methylene blue, and second, nitroanthraquinone which has recently been introduced by Bieling (11) into the field of experimental medicine. This latter indicator, which has only a light yellowish color, is

[2] Blood examined microscopically from guinea pigs that died within a few minutes from an overdose of ink showed no carbon particles. Also the observation of the eye in the albino guinea pig during ink injection showed only a blackening for a few seconds while the carbon passed through the capillaries of the retina.

TABLE III.

Cell Respiration of the Liver and the Spleen in Blocked Guinea Pigs Measured by the Methylene Blue Test.

Guinea pig No.	Liver cells. (Degree of reduction after various periods of incubation.)								
	10 min.	15 min.	20 min.	25 min.	30 min.	45 min.	50 min.	60 min.	90 min.
1 and 2	++	+++	++++	++++	++++	++++	++++	++++	++++
3 " 4	++	+++	++++	++++	++++	++++	++++	++++	++++
5 " 6	++	++	++++	++++	++++	++++	++++	++++	++++
7 " 8	++	++	++++	++++	++++	++++	++++	++++	++++
9 " 10	−	−	+	++	+++	++++	++++	++++	++++
11 " 12	−	−	−	+	++	+++	++++	++++	++++
13 " 14	−	−	−	−	+	+	++	++	+++
15 " 16	−	−	−	−	+	+	++	++	+++
17 " 18	−	−	−	+	+++	+++	++++	++++	++++
19 " 20	+	+++	++++	++++	++++	++++	++++	++++	++++
	Spleen cells. (Degree of reduction after various periods of incubation.)								
1 and 2	+++	++++	++++	++++	++++	++++	++++	++++	++++
3 " 4	+++	++++	++++	++++	++++	++++	++++	++++	++++
5 " 6	+++	++++	++++	++++	++++	++++	++++	++++	++++
7 " 8	+	+++	++++	++++	++++	++++	++++	++++	++++
9 " 10	−	++	++++	++++	++++	++++	++++	++++	++++
11 " 12	−	−	−	−	−	−	+	++	+++
13 " 14	−	−	−	−	−	−	−	+	++
15 " 16	−	−	−	−	−	−	−	+	++
17 " 18	−	−	+	+++	++++	++++	++++	++++	++++
19 " 20	++	+++	++++	++++	++++	++++	++++	++++	++++

−, no decolorization.
+, beginning decolorization at the bottom of the tube.
++, partial decolorization, extending to about one-half of the column of fluid in the tube.
+++, almost complete decolorization, extending to about three-fourths of the column of fluid in the tube.
++++, complete decolorization.
Incubation at 37°C.

transformed by the respiration of the living cells into a red amino compound.[3] It has the advantage that the medium need not be covered with mineral oil in order to exclude the oxygen of air since this reaction is not reversible. Furthermore, it allows an expression in quantitative terms by comparison of the test-tubes with a set of standard tubes that contain the amino compound in different amounts. From former work it was known that spleen and liver were the most important organs to be considered for this investigation. The technique of this experiment was as follows:

The same animals that were used for the complement titrations, described above, were killed at the time of the bleeding. Emulsions were made from the two spleens and two livers of duplicate animals by grinding the two kinds of organs separately with sterile ground glass in a sterile mortar, and suspending 2 gm. of these organs, respectively, in 10 cc. of physiological salt solution. 0.5 cc. of cell suspension was mixed with 0.5 cc. of salt solution and 0.1 cc. of a 1:1500 methylene blue dilution was added. This mixture was covered with 0.5 cc. of mineral oil, the tubes were incubated at 37°C., and readings made at different times to determine the degree of reduction. The results obtained with the methylene blue reduction test are tabulated in Table III.

In the nitroanthraquinone reduction test 3 cc. of the same cell suspension were mixed with 2 cc. of the indicator dilution 1:50 and the tubes were likewise incubated at 37°C. This test was interrupted after 3 hours incubation and the color changes were compared with a set of standard tubes of aminoanthraquinone. In order to obtain a clear color it was necessary to boil the cell suspension for a few minutes, for the purpose of precipitating the protein, and to test the filtrate. Former experiments had shown that short boiling did not change the color. All readings were made with the Walpole comparator, the object being to find a color among the standard indicator dilutions that would match the color of the test-tube. The scale of standard dilutions of aminoanthraquinone was prepared from a 1:400 stock solution by making decreasing amounts of this stock solution up to 10 cc. with physiological salt solution. The following scale was obtained in this way:

Scale of Standard Dilutions of Aminoanthraquinone.

No.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Indicator solution 1:400...	2	1.5	1	0.8	0.6	0.5	0.4	0.3	0.2	0.1	0.05	0.02
Physiological salt solution.	8	8.5	9	9.2	9.4	9.5	9.6	9.7	9.8	9.9	9.95	9.98

[3] Both nitroanthraquinone and aminoanthraquinone were procured from Meister, Lucius, and Bruening, Hoechst on the Main.

Table IV gives information on the results obtained with this test. Chart 2 illustrates the combined results of Tables III and IV.

It is of interest to note that with both tests, the methylene blue reduction test and the nitroanthraquinone reduction test, similar results were obtained. Assuming that cell respiration is an index of the vitality of the organs, especially of the more highly differentiated parenchyma, it would appear that within the 1st hour after the intravenous injection of ink, a marked depression of the cell vitality occurred which remained practically at this low level for about 8 hours. There followed a quick physiological recovery of the cells, however, as evidenced by the fact that 1 day after the ink injection a marked acceleration in the rate of reduction took place. It is of special interest to note that this functional restoration to physiological integrity is accomplished before signs of morphological regeneration are microscopically visible.

TABLE IV.

Cell Respiration of the Liver and Spleen in Blocked Guinea Pigs Measured by the Nitroanthraquinone Test.

Guinea pig No.	Degree of reduction after 3 hrs. incubation expressed in terms of standard dilution of aminoanthraquinone.	
	Liver cells.	Spleen cells.
1 and 2	IV	VIII
3 " 4	V	VII
5 " 6	IV	VIII
7 " 8	VII	X
9 " 10	VII	X
11 " 12	VII	X
13 " 14	VIII	XI
15 " 16	VIII	XI
17 " 18	VI	X
19 " 20	V	VIII

Provided that cell respiration runs parallel with other physiological functions of cell metabolism, the above experiments suggest that the blocking effect in guinea pigs of one intravenous injection of India ink is only partial. It is, moreover, only of limited duration, as the initial temporary disturbance of the cells of the reticulo-endothelial system

of the liver and spleen is followed by a regeneration setting in as early as from 1 to 2 days after the injection of the foreign substance.

In judging the efficacy of different substances in establishing a blockade, two qualities must obviously be taken into consideration: Such a substance should be of a proper dispersion in order to diffuse, and of such nature as to remain in the cells for a longer period. These experiments were carried out under optimal conditions for the reten-

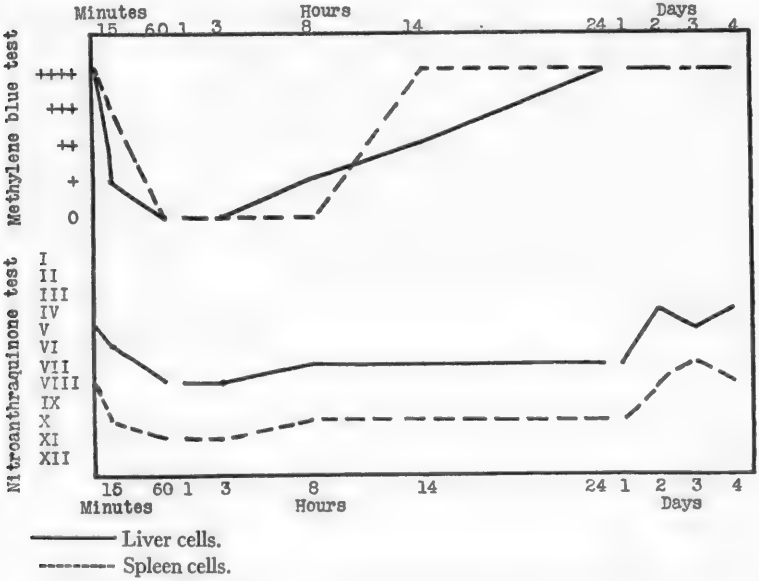

CHART 2. Results of the methylene blue test based on reduction values obtained after 25 minutes incubation at 37°C.

Results of the nitroanthraquinone test based on reduction values obtained after 3 hours incubation at 37°C.

tion of the injected material, since the carbon deposited in the cells stays there for a long time, whereas other materials, such as some dyestuffs and the colloidal salts of heavy metals, used for blocking, are excreted more rapidly.

Comparison of the curve of complement titer with the respiration values obtained with the reduction tests after the same intervals of time, shows that they follow the same general course. The signifi-

cance of all these observations as they affect hypotheses concerning phenomena of adsorption or the origin of complement from the reticulo-endothelial tissue of the spleen and liver, or from the leucocytes, is not yet clear.

SUMMARY AND CONCLUSIONS.

1. Intravenous injections of India ink into guinea pigs caused a decided drop in the complement titer which set in as early as 15 minutes after the injection, but did not reach its maximum for 3 hours. This drop was followed by a return to normal within the first 24 hours following the injection.

2. India ink mixed *in vitro* with guinea pig serum adsorbs the complement almost immediately to its full extent.

3. By means of reduction tests (methylene blue and nitroanthraquinone) it was shown that the respiration of the cells of the liver and spleen of guinea pigs was markedly impaired for the first 8 hours, following an intravenous injection of ink. Evidences of a return to normal functional vitality, however, became apparent by the end of the 1st day after the injection.

BIBLIOGRAPHY.

1. Siegmund, H., *Klin. Woch.*, 1922, i, 2566.
2. Hammarsten, O., Lehrbuch der physiologischen Chemie, Wiesbaden, 8th edition, 1914, 47.
3. Bechhold, H., Die Kolloide in Biologie und Medizin, Dresden, 4th edition, 1922, 213, 214.
4. Sachs, H., Hämolysine des Blutserums, in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 870.
5. Olsen, O., *Biochem. Z.*, 1921, cxxiv, 119.
6. Klopstock, F., *Biochem. Z.*, 1924, cxlix, 331.
7. Landsteiner, K., and Stanković, R., *Centr. Bakt., 1. Abt., Orig.*, 1906, xlii, 353.
8. Loewit, M., Infektion und Immunität, Berlin and Vienna, 1921, 190.
9. Jungeblut, C. W., and Berlot, J. A., *J. Exp. Med.*, 1926, xliii, 613.
10. Aschoff, L., *Ergebn. inn. Med. u. Kinderheilk.*, 1924, xxvi, 1.
11. Bieling, R., *Centr. Bakt., 1. Abt., Orig.*, 1923, xc, 49; *Z. Hyg. u. Infectionskrankh.*, 1923, c, 270.

MELANOMA (SARCOMA) OF THE EYE IN A SYPHILITIC RABBIT.

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PLATES 29 AND 30.

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Several years ago, we reported the occurrence of a malignant tumor which arose at the site of a syphilitic scar in the scrotum of a rabbit. This tumor was transplanted and subsequently used in an extensive series of investigations dealing with the subjects of tumor growth and malignancy. More recently, we have encountered a tumor of the eye in a rabbit a report of which is given in the present paper on account of the circumstances attending its development and the unusual character of the growth.

History.

The history of the tumor is as follows:

The rabbit was a male of the common grayish brown type, probably about 8 to 10 months old when first placed under observation. At this time, it was noted that the animal was apparently blind in the right eye. The pupil was fixed and irregular, and there was a diffuse opaque white mottling in the pupillary area. An ophthalmoscopic examination was not made.

On January 13, 1921, the animal was inoculated in the right testicle with a strain of *Treponema pallidum* (Strain V of our series) that had been carried in rabbits for 15 months and was of comparatively low virulence. An orchitis developed within 8 to 10 days and the inoculated testicle was removed (under ether anesthesia) on the 15th day after inoculation. A metastatic orchitis of the left testicle developed 40 days after inoculation, and about 2 weeks later there were a few papular lesions in the left scrotum associated with a general enlargement and induration of the inguinal and popliteal lymph nodes. The testicular lesion was comparatively slight and soon healed, leaving the testicle atrophic and fibrous. The papules in the scrotum persisted for several months with varying activity. At times, they almost disappeared, then recurred, and on several occasions showed a tendency to fuse, forming patches of infiltration with a scaly or weeping surface.

There were a few small lesions of similar character on the sheath and about the anus. These may or may not have been syphilitic. No other lesions were noted.

On February 6, 1922, the rabbit was reinoculated in the skin of the sheath with the same strain of *Treponema pallidum* used for the first inoculation, and on March 20, 1922, it was given a third inoculation. In this instance, the organism used was the Nichols strain. The local inflammatory reaction following these two inoculations was comparatively slight. No chancre developed, but on May 4, 45 days after the last inoculation, the right eye showed an intense congestion and edema of the conjunctiva, a diffuse clouding of the cornea with pannus, and small, pale, yellow masses in the upper anterior quadrant of the iris which protruded slightly into the anterior chamber; the bulb was tense and showed several irregular prominences in the region of the sclerocorneal junction. In addition, there was profuse lacrymation and pronounced photophobia. This condition persisted, with some intensification, for 5 or 6 days. The inflammatory reaction then began to subside and by the end of the 8th day the intensely acute manifestations had practically disappeared. The sclera was then of a distinctly yellow color, the bulb less tense and irregular than it had been, but the mass in the iris and ciliary body had extended forward and almost filled the anterior chamber, completely obscuring the greater part of the iris. The animal was in good physical condition and was killed for postmortem examination on the 9th day after the lesion of the eye was first noted.

Postmortem Examination.

The right eye was removed and sectioned horizontally. In the position of the iris and ciliary body there was a mass of tissue measuring 4.0 to 6.0 mm. in thickness which protruded into the anterior chamber (Fig. 1). The mass was semitranslucent and of a pale, yellowish pink color with irregular areas of black pigmentation. Its anterior limits were fairly distinct, but posteriorly it became indistinguishable from the vitreous. At its center, the growth completely surrounded the lens and laterally it fused with the cornea and the choroid. The cornea was thickened and clouded throughout; at the periphery it measured 4.0 mm. in thickness, while toward the center it diminished to 2.0 to 2.5 mm. The lens itself was translucent, but on the inner surface of the capsule there was a thin deposit of chalky white material which covered the pupillary area.

The heart, lungs, and liver were normal. The thymus and spleen were small but otherwise normal. The kidneys showed a moderate scarring. The left testicle was atrophic and its tunics were thickened. The brain and cord were normal. The thyroid was small and of a

yellowish gray color. The parathyroids and the suprarenals were considerably enlarged. The lymph nodes, with the exception of the deep cervicals, were very small and pigmented. The pineal gland was large. The hypophysis was normal. The bones and bone marrow were normal with the exception of an enlargement of the distal end of the right femur and some inflammatory thickening of the tissues about the knee. There was a mucopurulent exudate in the nasal passages and sinuses, and pus in both middle ears.

Demonstration of Treponemata.

Material from the lesion of the right eye was examined for treponemata by dark-field illumination with negative results. An emulsion was then prepared and 0.4 cc. was injected into the right testicle of three rabbits and a small piece of the cornea was implanted in the left scrotum of each of these animals. Two other rabbits were inoculated in the right testicle with an emulsion of a popliteal lymph node.

After a prolonged incubation period, all of these rabbits developed a syphilitic infection in the inoculated testicle which pursued an uneventful course. There was, however, no definite reaction at the site of the corneal implants. In one animal, a small translucent nodule developed and then retrogressed but the nature of the lesion was not determined. The animals were discarded at the end of about 4 months, all lesions having disappeared.

Microscopic Examination.

Microscopic examination of sections from the right eye showed an extensive pigmented growth composed chiefly of spindle cells (Figs. 1 to 7). The iris and ciliary body were virtually replaced by a growth which extended forward into the anterior chamber and the cornea and backward over the posterior surface of the iris, partially surrounding the lens, and into the choroid. The sclera was invaded in several places, but apparently the growth was still confined within the bulb except over a small area on the anterior or nasal side of the eye where it had penetrated into the conjunctival tissues at the sclerocorneal junction (Fig. 1).

The structure of the growth varied in different locations. On the anterior or nasal side, the growth in the ciliary body and adjacent parts

of the iris, the choroid, and the cornea was composed of small spindle cells arranged in irregular compact bundles with a fairly abundant stroma of dense fibrous tissue (Fig. 2). There was little or no pigment in this part of the growth and comparatively few cells showed mitotic division.

The growth in the iris was also composed chiefly of spindle cells, but the cells were much larger and more irregular and were arranged in bundles which showed a tendency to cross at right angles to one another as in the case of the muscle bundles of the iris (Fig. 3). Many of these cells showed mitotic figures.

The growth in the iris also contained more pigment than that elsewhere. The pigment was irregularly distributed but, in general, followed the outlines of what was the posterior surface of the iris and ciliary processes with a finer network extending through all parts of the growth (Fig. 1). It was both free in the tissues and contained in cells, some of which were round or oval, and closely resembled the normal pigment cells of the iris or of the retina, while others were irregular polyhedral or spindle shape (Fig. 3).

The cells composing the growth toward the periphery of the iris and in adjacent parts of the ciliary body were extremely irregular. They varied from a small to a very large spindle and from a small round cell to a large round or polyhedral cell with single or multiple nuclei (Figs. 4 and 5). Another feature of this part of the growth was the presence of numbers of giant cells or irregular protoplasmic masses many of which showed atypical mitotic figures.

In the choroid, large round or oval cells predominated. In fact, this type of cell was most numerous in all of the actively growing parts of the lesion with the exception of the cornea. Here, the growth was composed chiefly of oval and spindle cells (Fig. 6) surrounded by a rich network of coarse, wavy fibrils, and was practically devoid of pigment. The cells showed a remarkable uniformity of arrangement with the formation of a structure which, on section, presented an appearance which might be described as a pennate or even a herring bone arrangement.

There was no unusual development of blood vessels in any part of the growth and no hemorrhage. There were only two extensive areas of necrosis; one of these was situated on the anterior surface of

the iris and the other immediately in front of the lens. Over the posterior surface of the iris and in the region of the ora serrata, in particular, there was some diffuse necrosis, but elsewhere the cells were well preserved and in an active state of division.

There was practically no reaction in the tissues surrounding the growth except in the cornea, which showed a chronic inflammatory reaction (Figs. 6 and 7) identical in all respects with that seen in cases of syphilitic keratitis (Fig. 7). The reaction was most marked at the periphery of the cornea and in the region of the sclerocorneal junction, but, as is usual in such cases, there was a pronounced edema of the entire cornea with a slight cellular infiltration and a diffuse growth of capillary vessels.

DISCUSSION AND CONCLUSIONS.

It is evident from the description that has been given that the growth in the eye of this rabbit was a neoplasm of the type usually designated as a melanoma or sarcoma. Aside from the fact that the tumor appears to be the first of its kind that has been reported in a rabbit, the points of especial interest are its origin at the site of an old lesion of unknown etiology, the sudden assumption of extremely rapid growth, and the relation of the tumor to a syphilitic infection.

The evidence available indicates that the tumor originated in the periphery of the iris or in the ciliary body and that its occurrence was influenced in some way by a preexisting abnormality of a type that is comparatively common among rabbits. The time at which the neoplastic transformation took place is, of course, unknown, but the animals of this group had been examined regularly at least two or three times a week, with especial attention given to the eyes. It is not likely, therefore, that any decided reaction in the eye of this animal could have been overlooked, and it is almost certain that the time between the beginning of active growth and the detection of the lesion was only a few days. In fact, the sudden development of the lesion at an interval of 6 weeks after a superinoculation with a highly virulent strain of *Treponema pallidum*, the appearance of the lesion, and the occurrence of spontaneous retrogression at the end of a week did not suggest tumor growth but a pronounced case of granulomatous

syphilitic iridocyclitis. The condition was unusual, however, in so far as the excessive growth of tissue was concerned, and was of especial interest to us on account of its relation to a superinoculation. At the time, it was thought that the lesion might be an expression of allergy, and the animal was killed in order to obtain material for pathological study and with a view to establishing the supposed syphilitic nature of the affection. The failure to find treponemata by dark-field examination could be satisfactorily explained either on the basis of allergy or as a result of spontaneous resolution. The inoculation of other rabbits showed that treponemata were present, thus confirming the clinical diagnosis of syphilis. There was considerable delay in obtaining sections and the matter was dismissed until very recently when we had occasion to refer to the sections from this autopsy. It was then found that, while there was an unmistakable syphilitic reaction in the cornea, the main lesion was not syphilitic but a definite neoplasm.

These circumstances are related for the purpose of making clear the close association of what was undoubtedly an allergic reaction to syphilis and the sudden development of neoplastic activity. Both of these conditions appear to have been influenced by a preexisting non-specific lesion. Moreover, the occurrence of the two reactions coincided so closely that they appeared to be parts of a single process. This may have been merely a fortuitous coincidence but both conditions are so unusual that we are inclined to regard their simultaneous occurrence as evidence of an etiologic relation. Either condition may have influenced the other, but it seems likely that the original syphilitic infection was the factor which determined the allergic reaction to a subsequent inoculation and was in some way responsible for the sudden development of the tumor.

SUMMARY.

A melanotic tumor developed in a defective eye of a syphilitic rabbit following repeated genital inoculations with *Treponema pallidum*. The appearance of the tumor coincided with the development of a syphilitic lesion in the same eye, suggesting a relation between the occurrence of the two lesions.

EXPLANATION OF PLATES.

PLATE 29.

FIG. 1. Horizontal section through the middle of the eye reconstructed by joining up nasal and temporal portions. The areas designated by letters are those from which Figs. 2 to 7 were taken. × 7.

FIG. 2. Small spindle cell growth from *A*, Fig. 1. × 210.

FIG. 3. Large spindle cell growth from the iris (*B*, Fig. 1). × 210.

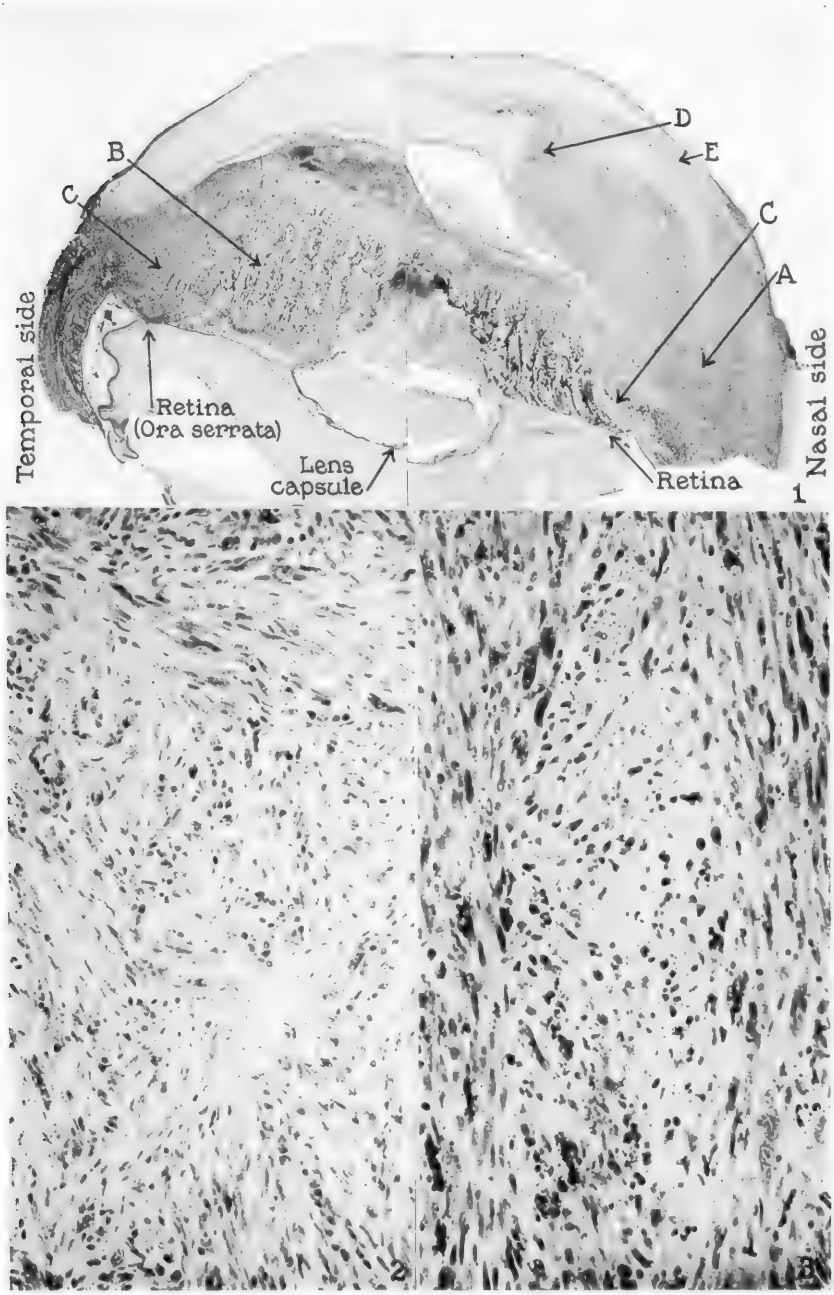
PLATE 30.

FIG. 4. Ciliary body or iris (*C*, Fig. 1). × 500.

FIG. 5. Same as Fig. 4. × 500.

FIG. 6. The tumor invading the cornea (*D*, Fig. 1). Note character and peculiar arrangement of tumor cells and the reaction in the cornea. × 210.

FIG. 7. Small spindle cell tumor growth with chronic inflammatory reaction (syphilitic) in the peripheral and superficial parts of the cornea (*E*, Fig. 1). × 210.



(Brown and Pearce: Melanoma of eye in syphilitic rabbit.)

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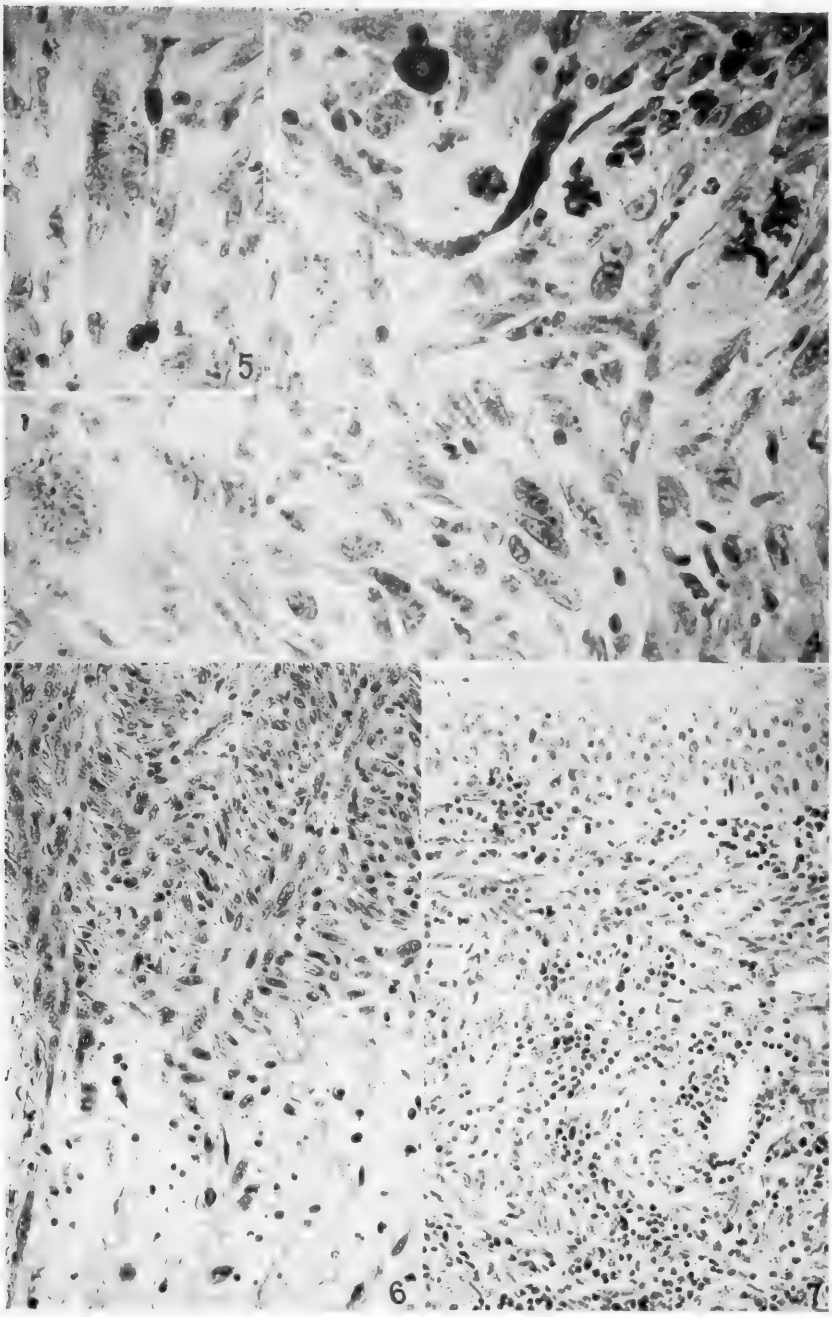

(Brown and Pearce: Melanoma of eye in syphilitic rabbit.)

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THE CULTIVATION OF THE GRANULES OF VACCINIA VIRUS.

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An attempt was made in the following experiments to understand more thoroughly the nature of vaccinia virus. The literature has been increased yearly with contradictory ideas and theories as to the cause of the potency of the virus, yet no one has been able to produce with certainty this potent factor in unadulterated form. Some have succeeded in passing a potent virus through Berkefeld filters under special conditions, while most authors have failed. Others have concerned themselves especially with efforts to recognize in certain granules a visible virus.

Upon the suggestion of Dr. W. G. MacCallum, we endeavored to maintain the rôle of the so called "granules," seen in tissues and smears from vaccinia and smallpox by Calmette and Guérin (1), Prowazek (2), Paschen (3), Hallenberger (4), and many later observers, and which appear in such myriads in vaccinia virus. These "granules" are easily recognized by dark-field illumination. It was definitely ascertained that whenever sufficient numbers of "granules" were seen in material separated from the whole vaccine by various methods, it would give a typical vaccinia lesion on the scarified cornea of a rabbit. When no "granules" were seen, or when present in very small numbers, no lesion occurred after similar inoculation of the material.

As the "granules" seemed to form such an important part of the virus, an attempt was made to grow them (using as a control the fluid part of the vaccine) in tissue cultures, because of the successful growth of the whole vaccinia virus in connection with living tissue both *in vivo* and *in vitro*. *In vivo*, it has been cultivated in other tissues besides the skin.

Noguchi (5) succeeded in passing the virus from one rabbit to another by intratesticular inoculations. The brain, too, presents a good medium, according to Marie (6), Levaditi, Harvier, and Nicolau (7). Furthermore, Levaditi and Nicolau (8) showed that the virus will grow in epithelial tumors although not in connective tissue tumors. *In vitro*, it has been grown successfully by several groups of workers. Steinhardt, Israeli, and Lambert (9) used cornea immersed for a short time in dialyzed virus, while Gins (10), Parker (11), and Hach (12) used respectively cornea, testis, testis and spleen of already infected animals. Parker and Nye (13) grew normal testis in the plasma of an infected animal whose blood is virulent for a period of time as shown by Levaditi (14) and Ohtawara (15).

Procedure.

The "granules" were obtained in as pure a state as possible by separating them from glycerolated vaccinia calf lymph (kindly sent us by Dr. F. M. Huntoon of the H. K. Mulford Laboratories) according to the differential centrifugalization method of MacCallum and Oppenheimer (16) with sterile technique. The virus was always centrifugalized at rapid speed for 40 to 60 minutes. About 0.1 cc. of the top layer was carefully removed without disturbing the contents of the lower part of the small tube. This was transferred to another tube and sterile Locke solution added. After mixing thoroughly, the tube was centrifugalized for 20 minutes and the "granules" thrown to the bottom in a solid layer. The supernatant fluid was all removed, and this was the material used as the control. It consisted of the serum part of the virus, glycerol, and Locke solution. The "granules" were subsequently washed twice more in a similar fashion with Locke solution, the resulting supernatant fluid being entirely discarded after each centrifugalization. The final "granules" obtained were diluted to their original concentration in 0.1 cc. Locke solution. This was the material called by us "washed granules," which we endeavored to grow. When examined under the dark-field microscope, it showed a mass of tiny granules, moving with what appeared to be Brownian movement. No tissue fragments or bacteria were seen as in the whole virus by this method.

The aim of our technique was to use only sterile materials, necessitating our using only embryonic tissues. These also seemed superior because of the facility with which they grew. At first we used embryonic rabbit cornea in homologous iced plasma. The latter we changed for the heparin plasma of Craciun (17). Even though we obtained pure epithelial growth, we could not avail ourselves of rabbit tissue because it was difficult and expensive to get rabbits far enough advanced in pregnancy to furnish embryos with large corneæ, and because without the time-consuming operation of making a skin incision to examine the mammary glands, it was impossible to differentiate with certainty pregnant from non-pregnant animals. We, therefore, changed to the guinea pig which proved to be an ideal animal. The size of its embryos could be ascertained easily by palpation

and we could, therefore, always get sufficient embryonic cornea. With this tissue we used homologous heparin plasma, prepared in advance in ampoules. We always obtained enough cultures with pure epithelial growth to continue transferring them throughout the series. All cultures contaminated with bacteria were immediately discarded.

The embryonic cornea was cut into tiny pieces in Locke solution. The main cultures were made with cornea, Locke solution, plasma, and a tiny drop of "washed granules" from the tip of the smallest possible capillary pipette. Other cultures consisted of cornea, plasma, and "control material." Here no Locke solution was added as it was already contained in the material. To control the materials used, cultures were made with pieces of cornea, Locke solution, and plasma. Most of the cultures were made on cover-slips which were inverted and sealed over rather deep hollow, ground glass slides and incubated at 38°C. In a few series the Carrel D flasks and the Gabritschewsky dishes were used. Every 3 to 7 days the cultures were washed in Locke solution and changed to a new medium of fresh cornea and plasma. In the intervals, that is every 2 or 3 days, the fluid part of the cultures was pipetted off and the cultures received a fresh drop of plasma. About every 8 days the presence of the virus was tested by inoculation on a scarified rabbit's cornea. The "washed granules" and "control material" used for the series were, of course, similarly tested beforehand, the "granules" always and the "control material" never giving the vaccinia lesion.

Two different types of control series were made. From each set of cultures several were set aside in the incubator in their original state and never transferred to a new medium. These were inoculated at the same time as the last transferred cultures of the series. This served to see if "granules" or "control material," if suitably incubated, could live or multiply in connection with cultures that were no longer living. Some cultures were allowed to remain untransferred for 2 weeks, others for 3 weeks, and then transferred every 7 days, to discover at what period the "granules" died. It was found unnecessary to do this with the "control material."

Another control series was made to discover, if possible, the location of the "granules"—whether they are transferred from the old to the new tissue, or whether they merely remain alive in the plasma and will, therefore, disappear if the original piece of tissue is removed after being in contact with the new medium for a few days. The cultures were made as above and transferred to the new medium in 7 days. At this time a camera lucida sketch of the culture was made; the original inoculated piece of tissue was labeled "A" and the fresh piece of cornea (to which no "granules" were ever added) labeled "B." At the end of 7 days, both "A" and "B" were removed, washed separately in Locke solution, and each added to a new medium of fresh cornea and plasma—thus giving rise to an "A" and "B" subdivision of the same series. Both of these branches were treated as always about every 7 days, and each inoculated separately to test for the presence of the vaccine.

RESULTS.

Table I gives the results typical of each of the examples described above. 722 cultures were made in all. There were twenty-six series, some of them including several of the above types of experiments.

TABLE I.

Series No.	Age of culture when tested.	No. of transfers.	No. of cultures inoculated.	Material in culture.	Result.
	days				
IV	7	0	4	Granules.	Negative.
IV	13	1	5	"	"
IV	21	2	4	"	Positive.
XX	26	4	3	"	"
XX	25	0	7	"	Negative.
XXIII	21	2	3	"	"
XXIII	71	9	2	"	Positive.
XXIV	28	3	3	"A"	"
XXIV	28	3	3	"B"	"
XXV	21	2	4	"A"	"
XXV	21	2	4	"B"	"
XXV	49	6	3	"A"	"
XXV	49	6	3	"B"	"
XXV	56	7	2	"A"	"
XXV	56	7	2	"B"	"
XXVI	17	2	5	Control material.	Negative.
XXVI	17	0	4	" "	"
XXVI	17	2	3	Granules.	"
XXVI	53	7	4	Control material.	"
XXVI	53	0	4	" "	"
XXVI	53	7	4	Granules.	Positive.
XXVII	34	4	4	"	"
XXVII	34	3*	4	"	Negative.
XXVII	34	2†	4	"	"

* Kept 14 days before making first transfer.
† Kept 21 days before making first transfer.

Table I shows that whereas the fluid content of vaccinia virus will not produce a lesion typical of vaccinia even after being in contact

with growing epithelial cultures (Series XXVI), the "granules" found in the virus not only remain alive for so long a time as 71 days (Series XXIII) in sufficient numbers to produce a lesion when inoculated, but must actually increase in potency in connection with the cultures. This is shown by the fact that cultures in the first transfer did not contain enough "granules" to give rise to the lesion, but after growing for a longer time, they would produce the typical corneal reaction (Series IV). Likewise, two cultures in the late transfers would give a lesion whereas in the second transfer of the same series four cultures were needed to produce a similar lesion (Series XXV). This can be readily explained by the gradual removal of the fluid part of the cultures which contained some inoculated "granules," leaving in the cultures only those "granules" already attached to the tissue. These were never present in sufficient numbers to produce a lesion until they grew for at least 21 days under the conditions of our experiments.

It was already known that only living cells will keep the virus alive. Indeed, Steinhardt and her coworkers found that if corneal tissue first be killed by freezing or treating with hypotonic salt solution, the virus will soon die out. Furthermore, one can conclude from the work of Williams and Flournoy (18), Krumbach (19), and Hirano (20) that only tissue which is actually growing will permit the maintenance of the virulence of the virus. From our experiments it is seen that the living growing cell is also absolutely necessary to the growth of the virus "granules." They always died when left with untransferred tissue cultures that had also died (Series XX).

The fact that the "B" series as well as the "A" series produced vaccinia lesions (Series XXIV and XXV) indicates that the "granules" must, in part, leave the older "A" tissue for the more favorable soil to be found in the fresher "B" tissue. This is plain from the fact that the plasma containing the inoculated "granules" was always washed away before the "A" was added to the "B" cornea. It is interesting to note that after inoculation on a rabbit's cornea, "B" cultures always had a shorter incubation period and gave a larger vaccinia lesion than did the "A" cultures. This indicated that the cells probably have to be in a fairly good condition because in our experiments no more virus was present in the cultures in which neither fresh plasma nor new tissue was added until after 2 weeks and which therefore did

not show any further growth when transferred after this lapse of time (Series XXVII). But it was still present, even though weakened, in the "A" pieces which received new medium four or five times in the meanwhile and were situated near the fresh "B" tissue.

We have from these studies no morphological proof of an increase in the number of the "granules" since they cannot readily be distinguished from other granules normally seen in the cells of tissue cultures. But the experiments differ from others which have been concerned with the cultivation of the virus of vaccinia in that the "granules" have been washed free from all adhering fluid and then cultivated in increasing potency while the fluid in which they were formerly suspended shows no power to infect the rabbit's cornea even after cultivation. They show at least that the power of the vaccine to infect an animal is inherent in the granules even though further analysis of their nature must be deferred.

SUMMARY.

The technique is described whereby vaccinia "granules" are separated from all other material of glycerolated vaccinia calf lymph and cultivated *in vitro* with embryonic tissues.

These "granules" remain alive, as tested by rabbit corneal inoculation, for as long as 71 days, when grown in connection with growing tissue; they fail to remain potent if cultivated with dying cells, showing in this way the same characteristics as whole vaccine virus. The potency of the "granules" increases with the age of the culture under the first mentioned conditions.

The fluid part of the vaccinia lymph which remains after the removal of all "granules," is impotent under all conditions.

BIBLIOGRAPHY.

1. Calmette, A., and Guérin, C., *Ann. Inst. Pasteur*, 1901, xv, 161.
2. Prowazek, S., *Arb. k. Gsndhtsamte*, 1905, xxii, 535.
3. Paschen, E., *Centr. Bakt., 1. Abt., Ref.*, 1906, xli, 637.
4. Hallenberger, *Centr. Bakt., 1. Abt., Orig.*, 1917–18, lxxx, 89.
5. Noguchi, H., *J. Exp. Med.*, 1915, xxi, 539.
6. Marie, A., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 476.
7. Levaditi, C., Harvier, P., and Nicolau, S., *Compt. rend. Soc. biol.*, 1921, lxxxv, 345.

8. Levaditi, C., and Nicolau, S., *Ann. Inst. Pasteur*, 1923, xxxvii, 443.
9. Steinhardt, E., Israeli, C., and Lambert, R. A., *J. Infect. Dis.*, 1913, xiii, 294.
Steinhardt, E., and Lambert, R. A., *J. Infect. Dis.*, 1914, xiv, 87.
Steinhardt (Harde), E., and Grund, M., *J. Infect. Dis.*, 1915, xvi, 205.
(Steinhardt) Harde, E. S., *Compt. rend. Soc. biol.*, 1915, lxxviii, 545.
Harde, E. S., *Ann. Inst. Pasteur*, 1916, xxx, 299.
10. Gins, H. A., *Z. Hyg. u. Infectionskrankh.*, 1916, lxxxii, 89.
11. Parker, F., Jr., *J. Med. Research*, 1923–24, xliv, 645.
12. Hach, *Centr. Bakt., 1. Abt., Orig.*, 1925, xciv, 270.
13. Parker, F., Jr., and Nye, R. N., *Am. J. Path.*, 1925, i, 325.
14. Levaditi, C., *Compt. rend. Soc. biol.*, 1921, lxxxv, 425, 429.
15. Ohtawara, *Scient. Rep. Gov. Inst. Infect. Dis.*, Tokyo, 1922, i, 203.
16. MacCallum, W. G., and Oppenheimer, E. H., *J. Am. Med. Assn.*, 1922, lxxviii, 410.
17. Craciun, E. C., *Bull. Johns Hopkins Hosp.*, 1925, xxxvii, 428.
18. Williams, A. W., and Flournoy, T., *Studies from The Rockefeller Institute for Medical Research*, 1905, iii.
19. Krumbach, H., *Z. Immunitätsforsch., Orig.*, 1922, xxxiv, 477.
20. Hirano, N., *Am. J. Path.*, 1925, i, 635.

THE PRESENCE OF DESQUAMATED ENDOTHELIAL CELLS, THE SO CALLED CLASMATOCYTES, IN NORMAL MAMMALIAN BLOOD.

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PLATES 31 AND 32.

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There has been increasing evidence that, under many abnormal conditions, the circulating blood contains the type of cell known as the endothelial phagocyte, the clasmatocyte, or the macrophage. The theory that endothelium gives rise to free, phagocytic, mononuclear cells was first advanced by Mallory (1), in 1898, in connection with the study of the lesions of typhoid fever. He found desquamated endothelial cells in clots in the heart's blood and said that it was difficult to explain why they had not been seen in the peripheral blood at some stage of the fever, unless they were destroyed by the technique of making the films (page 627). The first observations we have found of this cell in the peripheral blood were by Rowley (2), who found large cells with distinctive nuclei and a cytoplasm somewhat similar to that of the large lymphocyte in the blood of a patient with tertian malaria and in three cases of lymphatic leucemia, and described them so accurately as to give little difficulty in the interpretation. She found them containing both red and white cells. The next year (3) she described watching remarkable phagocytosis of red and white cells by large, living cells from the blood in a case of fatal anemia. In 1911, Leede (4) described very delicate lymphocytes in the blood of a case of ulcerative endocarditis. Again, the interpretation that he was dealing with endothelium is ours. Neukirch (5) made the first interpretation, of which we are aware, of endothelium in the peripheral blood, in cells containing bile pigment, from a patient with jaundice. Endothelium was then found in the blood by Kraus (6) in a case of splenomegaly. In 1913, Netoušek (7) described endothelium in the circulating blood of a case of cholera and subsequently (8) in a variety of pathological conditions, ulcerative endocarditis, leucemia, chlorosis, in staphylococcus infections, in typhoid fever, after typhoid inoculations, and in cancer. In this second communication he stated incidentally that he had seen cells in normal blood, which he suspected were also desquamated endothelium. Křiženecký (9) studied the blood of frogs after a ligature had remained around one leg for an hour, and found desquamation of sheets of endothelium into the blood stream. Since then, endothelium has been seen by many observers in the blood, most often in ulcerative endocarditis and in leucemia. Kaznelson (10) found

numerous endothelial cells in the blood at certain times in cases of purpura. In our interpretation, it was endothelium which was described by Franco and Ferrata (11) in leucemic blood, and illustrated by Ferrata (12, Plate xx) under the name of "hemohistioblast." They, however, consider the hemohistioblast as a cell of the connective tissues and think that it can give rise to neutrophilic and eosinophilic leucocytes. In 1919, Schilling (13) reported two cases of ulcerative endocarditis in which he found a great increase both in monocytes and in endothelial cells, and the latter showed the same remarkable phagocytosis of whole cells as the case reported previously by Miss Rowley. Schilling interpreted his findings as showing the genetic relationship of monocytes and endothelial phagocytes and their separation from the myeloid and lymphoid forms. Further observations and analyses of the literature have been given by Bittorf (14), who discusses desquamation of endothelium in relation to hemorrhagic diathesis, and by Richter (15), who found the cell, which we are describing as endothelium, in in a case of leucemia and interpreted it as Ferrata's hemohistioblast.

We propose to show that desquamated endothelium is a practically constant constituent of normal blood, that it is not identical with the monocyte, and that its numbers are increased under many pathological conditions. Thus, we think that there are two strains of phagocytic mononuclear cells in normal blood, desquamated endothelium and monocytes.

It must, however, be admitted that there is a marked tendency at the present time toward the theory that the mononuclear cells of the connective tissues represent a single strain, which is more and more coming to be regarded as the type of cell which the experiments of Goldmann (16, 17) discriminated as a highly phagocytic type, the macrophage of the connective tissues. The essential unity of the phagocytic mononuclear cells as a group derived from endothelium is primarily associated with the name of Mallory. It is this same concept which seemed involved in the original use of the term "reticulo-endothelial system" by Aschoff (Aschoff and Kiyono, 18, page 386), but in his most recent paper Aschoff (19, 1926) brings out a point which agrees with our experience that there is a physiological distinction between the reticular cells and the reticulo-endothelium of the sinuses of lymph glands because the reticular cells take in particulate matter, both more slowly and in less amount.

Cunningham, Sabin, and Doan (20–22) have recently advanced the theory that the phagocytic mononuclear cells of the connective tissues are representatives of two different strains of cells: one, the clasmatocyte, probably derived from endothelium, and the other, the monocyte, derived more directly from mesenchyme. The discrimination of these two strains of cells is largely on the basis of a difference in cytoplasmic activity, as judged by the supravital technique. By them the clasmatocytes are to be identified, as is generally accepted with the pyrrol cells of

Goldmann (16, 17), the macrophages of Metschnikoff (23), of Evans and Schulemann (24), and of Evans (25). They are the cells which take in the largest amounts of the insoluble dyes of our experiments and segregate them in large masses in vacuoles of digestion. We think that these vacuoles of digestion form only after materials have been taken into the cell, so that the appearance of the cell varies greatly, according to its functional state; and that the vacuoles, when formed, are distributed throughout the cell, entirely without pattern. In the monocyte, on the other hand, Cunningham, Sabin, and Doan found that there is a special constant organ of the cytoplasm, namely, a rosette of tiny bodies, probably small vacuoles, which stain in a fairly constant shade in neutral red and surround the centrosphere. It is clear that monocytes, as well as clasmatocytes, can phagocytize massive structures, such as whole red cells or white cells, but, on account of the presence of the rosette around the centrosphere, such phagocytized debris occupies a peripheral position in the monocyte.

The most important development of this concept that there is a strain of cells of the connective tissues, characterized by a special rosette, is the identification of the epithelioid cell, as it occurs in tuberculosis and other pathological conditions, such as Hodgkin's disease, as a derivative of the monocyte (22, 26). The epithelioid cell is considered in these two papers as a type in which there has been an enormous increase in the size of the rosette, due to an actual decrease in the size, but a great increase in the number of the individual bodies of the rosette of the original monocyte. A further indication of the value of the concept of two different strains of phagocytic mononuclear cells is the theory that the two well known types of giant cells of the general connective tissues come from the two different strains of cells. A type of giant cells, with scattered nuclei, is readily produced from the Kupffer cells of the liver by repeated injections of any particulate matter. Such cells were illustrated by Evans, Bowman, and Winternitz (27) by injections of trypan blue and tubercle bacilli. We think that such giant cells may come from clasmatocytes. The second type, the typical giant cell of tuberculosis, has its nuclei placed peripherally, owing, we think, to the central position of the hypertrophied rosette of the original monocyte. It is our theory that the constant presence of the rosette in the normal monocyte indicates that this cell has a different function from the clasmatocyte in connection with dealing with the physiological debris of the body, and that the marked development of the rosette in the epithelioid cell indicates a specific reaction of this cell under pathological conditions.

It is interesting to note that Kageyama (28), working in Aschoff's laboratory on experimental tuberculosis in mice, has found that the bacilli injected into the peritoneal cavity soon reach the sinuses of lymph glands, where they are phagocytized in large numbers by the endothelial cells, which round up, become free, and then disintegrate. The second reaction is the appearance of leucocytes which then take in the freed bacilli. This process may be repeated with the subsequent phagocytosis of both bacilli and leucocytes by other histiocytes. After 5 to 6

days, there is a third reaction, namely, a proliferation of cells in the follicles with the production of epithelioid cells. He did not find bacilli within these early epithelioid cells in contrast to the endothelium of the sinuses.

The validity of the concept that there are two different strains of phagocytic mononuclear cells was strongly called in question by the studies of M. R. Lewis (29) with tissue cultures of blood. Mrs. Lewis started by adding drops of blood to cultures of connective tissue to study the phagocytosis of the red blood cells by clasmatocytes, and then went on to follow hanging drops of blood alone. In these specimens, she demonstrated that in avian and in mammalian blood, two different strains of large phagocytic forms appeared, one with large vacuoles, diffusely scattered throughout the cell, without pattern, and the other with small vacuoles around the centrosphere, the typical epithelioid cell. These observations were extended to the blood of fishes and reptiles by Lewis and Lewis (30), and to the study of the epithelioid cells of tuberculosis by Lewis, Willis, and Lewis (31). Their conclusion was that the monocyte of the blood could be shown to give rise to both strains of the phagocytic mononuclear cells.

Sabin, Doan, and Cunningham (22) had also discriminated the monocytes from the clasmatocytes on the basis of a difference in origin, believing that the monocytes came from a reticular cell, that is to say, directly from mesenchyme outside the vessels, while the clasmatocytes could be more closely related to endothelium. In regard to the origin of the clasmatocytes from endothelium, there are two different groups of clasmatocytes to be considered: first, specific types that are intravascular, and second, the clasmatocytes or macrophages of the connective tissues. The intravascular cells are the Kupffer cells of the liver, and the corresponding cells which can be stimulated to form within the veins of the splenic pulp (22) and in sinuses of the bone marrow (Doan, Cunningham, and Sabin, 32). It is our opinion that the Kupffer cells of the sinuses of the liver are daughter endothelial cells, which have put out cytoplasmic processes exactly like the sprouts of the original angioblasts; and that by means of these cytoplasmic processes which attach to the opposite wall, the cell is held in midstream of the blood. Moreover, we think that the cells which can be experimentally produced in spleen and bone marrow are analogous to Kupffer cells, except that they do not attach themselves so relatively permanently to the inner walls of the veins of the pulp, but wander freely out into the spaces of the pulp of the spleen or the spaces of the bone marrow, or into the peripheral blood stream itself. Thus, the clasmatocytes of the liver, spleen, and bone marrow very probably arise intravascularly from the so called specific endothelia.

In regard to the origin of the other group of clasmatocytes, those of the diffuse connective tissues, it is by no means so easy to determine whether they are directly related to endothelium or not. It was shown by Goldmann that about half of the cells of the subcutaneous and intermuscular tissues are highly phagocytic. These cells are like the clasmatocytes from the specific endothelia, both in appearance and in function. They react in the same manner toward particulate matter

artificially introduced, and they take in red blood cells in the same manner. It is our belief that many of the new clasmatocytes in adult connective tissues arise from the division of preexisting clasmatocytes, but we have made certain observations that indicate that in the embryo they may be more closely related to the endothelium. In living blastoderms of chicks of the 4th to the 7th day of incubation, we have found irregular clumps of cells clinging to the outer walls of certain vessels which we have interpreted as indicating the developing of clasmatocytes from endothelium. However, Sugiyama (33), working with the same material, came to the conclusion that these adventitial cells come from mesenchyme, since they do not stain with neutral red until they become free, while the endothelium itself does react to the stain. It is believed by Mallory and his school that there is a formation of new clasmatocytes from the endothelium of vessels in the adult under many pathological stimuli. But it is clear that when these cells form along the outer wall of a vessel, there is always a possibility of two different interpretations concerning their origin, either from the division of an endothelial cell, or from mesenchyme in the adventitial position; and thus the evidence cannot be regarded as conclusive for either view.

Thus, while it has not been possible to bring convincing proof that endothelium may give rise to phagocytic types from its outer wall, it is not difficult to show that an endothelial cell, while forming the wall of a vessel, may become phagocytic, and that phagocytic types drop off into the lumen of the vessel. These observations were made by Maximow (34) for mammalian embryos in 1909 and his Fig. 4, on Plate xviii, shows both a red cell within an endothelial cell in the wall of a capillary and exactly similar cells in the lumen, which he interpreted as free, phagocytic endothelial cells. In 1922 Sabin (35) described watching the actual freeing of such cells from the inner wall of the endothelium of vessels in living specimens. She saw this process in the area vasculosa of chicks from the 3rd day of incubation on, and the same thing has now been seen in chicks of the 2nd day of incubation by Sugiyama. Both Maximow and Sabin interpreted these endothelial phagocytes as transitory in the blood stream, but in the light of the results of Lewis and Lewis with cultures of blood, we decided to give the matter of their possible permanence a thorough test. We began with the blood of adult pigeons and found that with the supravital technique we could readily find the type of cell which we interpret as desquamated endothelium, quite distinct from the monocyte. The studies which we are reporting here, however, have been made with the blood of normal and tubercular rabbits and with normal human blood.

We have found that there is a new type of cell, hitherto undescribed, as far as we are aware, as a practically constant constituent of normal blood. We consider that it belongs to the general strain of endothelial derivatives. This cell has been frequently seen under pathological conditions, when its numbers are increased. Such an increase is constant at a certain stage in experimental tuberculosis in rabbits, and our drawings are from the blood of tubercular, as well as normal rabbits. These cells appear in normal blood in a great variety of forms and sizes; merely for convenience of description, they are divided into four types, all of which, however, grade into each other with many transitions. These different forms are given in Figs. 1 to 12. The magnification of each cell is shown by the accompanying red cell.

There are, first, long cells, such as are shown in Figs. 1 to 3, which vary from cells like the one of Fig. 1, whose length is about $2\frac{1}{2}$ times the diameter of a red cell, to the long types of Figs. 2 and 3. The cells of Figs. 1 and 2 were from the blood of normal rabbits; that of Fig. 3 from a tubercular rabbit. We have also found these very long cells in normal human blood. The second group is made up of cells about the size of the small lymphocyte; they are not illustrated, for they are like the third group in every detail, except size. The third group is shown in Figs. 4, 5, and 7, and is made up of cells varying in size from that of an intermediate lymphocyte to that of the largest monocyte. Of this group, Fig. 4 was from a normal rabbit. The fourth group, Figs. 6, 8, 9, and 10, contains the largest types. In this group the cell of Fig. 8 was from a normal rabbit. Attention is called to the great size of the cell of Fig. 9, which has a length about 8 times the diameter of its red cell, which makes its greatest length about 40μ on the basis of the rabbit's red blood cell being 5 to 6μ in diameter. Such a cell we think entirely comparable to a giant Kupffer cell. It is the large cell which has been found in pathological conditions and which has been so frequently described. We have found it also in normal human blood, as well as in blood from the normal rabbit.

We have found, too, that these cells are characteristic both as to their nuclei and their cytoplasm. In form, the nuclei may be round, oval, long and narrow, or indented. The most obvious characteristic of the nucleus in vital preparations is the sharp, nuclear contour. This makes it look so much like the nucleus of fresh preparations after

the addition of a little acetic acid, that we have heretofore mistaken these cells for degenerating small and larger lymphocytes. The largest forms we have previously taken for disintegrating masses of platelets, with some degenerating cell clinging to them, to account for the nucleus.

In the supravital preparations, these cells must be discriminated from lymphocytes and from degenerating neutrophilic leucocytes, the so called "non-motile" cell of Sabin (36). The nuclei of the endothelial cells have an extremely large amount of chromatin which is finely divided and lacks the concentrations in the nodes, so characteristic of the nuclei of lymphocytes. In the living preparations, the nuclei look dark, on account of the amount of chromatin, in contrast to the pale and entirely structureless nuclei of the non-motile leucocytes. This will be clear from a comparison of the non-motile cell shown by Sabin (36, Fig. 2) with the cells of this paper. The characteristics of the nuclei of the desquamated endothelial cells are, however, brought out most clearly in fixed films and are shown in our Figs. 11 and 12 from cells of the blood in films, stained with Wright's blood stain, which are practically the same as the nuclei shown on Ferrata's colored plate (Figs. 1 to 4, on Plate xx, 12) and by all the cells of Richter (15). The chromatin of these cells is massive and, in our view, made of closely packed granules. We interpret many of the clear zones as interspaces; some of the nuclei have also definite nucleoli, as was shown by Ferrata and Richter. The characteristics of the nuclei are to be studied with care, since we find that the cytoplasm is so fragile that some of the cells of this type appear not infrequently as nuclei with thin, torn veils of cytoplasm around them.

The cytoplasm of these cells, especially of the long, the small, and the intermediate types, is entirely clear and structureless, except for certain refractile bodies. Of all the types, the small cells have fewest of the refractile bodies, there often being but five or six clinging to the nucleus, while the larger cells may be quite filled with them. The cytoplasm of more than half of the endothelial cells is entirely unstained in our supravital technique, when the blood is first drawn. These unstained forms are shown in Figs. 1 to 6. The refractile bodies are shown as either light or dark, as they actually appear, according to the focus at which they were viewed. They are irregular in size

and somewhat in shape; they are decidedly more refractile than mitochondria, which are rather dull in the living state, but they are less refractile than the granules of the non-motile cell of the supravital preparations. We have studied these cells in films with Janus green alone, according to the suggestion of Dr. W. H. Lewis, so that the combination of neutral red with Janus green might not obscure mitochondria, and when one of the endothelial cells lies close to a lymphocyte, the contrast is striking. The mitochondria of the lymphocytes are a clear blue color in Janus green, while the refractile bodies of the desquamated endothelial cells react, if at all, with a pale, greenish tint. This reaction we do not interpret as having any relation to mitochondria, and there are no other bodies to be seen in the cells. We wish to make it clear that by these observations we do not mean that the clasmatocytes always lack mitochondria, since Sabin, Doan, and Cunningham (22) have shown many clasmatocytes with mitochondria, but we do mean that many of the cells we are here describing as present in normal blood, and interpreting as desquamated endothelium, apparently lack them; in other words, we think that mitochondria may not be present continuously through the entire life cycle of a given cell.

When one of the unstained cells is watched, a staining of the refractile bodies may develop, or a cell, such as the one of Fig 9, may show a few stained bodies when it is first observed—immediately after being taken from the animal. In the cells of Figs. 7, 8, and 10, all the formed bodies in the cytoplasm were stained in neutral red. In some cells of this type, we have seen the scarlet reaction which neutral red takes in acid solution. Of the larger cells, one is shown, Fig. 6, in which there were no neutral red bodies, but the cell had phagocytized a red blood cell, which was placed against the nucleus of the original cell. The yellow color of the unchanged hemoglobin was entirely distinct in the specimen. The cell of Fig. 9 showed a few stained bodies, while that of Fig. 8 showed many. The sheet of endothelium shown in Fig. 10 was only partially stained when first found, but gradually all the formed bodies took the dye.

In the cells of Figs. 7 to 10, most of the stained bodies show a uniform staining with neutral red, but we have found endothelial cells in the blood, with stained vacuoles, reacting with every shade of neutral

red, so characteristic of the clasmatocytes in active function from the spleen and peritoneal exudates shown by Sabin, Doan, and Cunningham (22). We have not succeeded in getting a drawing of this type of the cell from the blood, on account of the rapid fading of the stain. We had the same experience in getting drawings of the highly phagocytic types for the previous paper, and had to make many attempts, with the artist ready to work before the preparations were made, in order to get any adequate record of the cell. In watching these large, functionally active cells in the supravital preparations, we have found that the color usually fades suddenly from the cell, leaving no trace; that is to say, the dye becomes entirely bleached out. In this process, the nucleus takes no stain. Nevertheless, the clasmatocyte is clearly dying at the time of the sudden bleaching, and rapidly degenerates and disintegrates. This is different from the way the leucocyte dies, for its nucleus begins to stain with the acid reaction of the dye. Doan (37) has followed the death and disintegration of highly phagocytic clasmatocytes of the bone marrow in supravital preparations, from cases of pernicious anemia, and has determined the rate of degeneration by successive differential counts of the same preparation. We, therefore, conclude that these cells frequently disintegrate at the end of a phase of digesting an enormous amount of debris. Exactly the same phenomenon of the bleaching of neutral red during cytolysis was observed by Plantin (38) and by Needham and Needham (39) with amebæ. Needham and Needham interpret this bleaching of netural red in their preparations as follows: that the cytolysis develops not only an acidity, but also a reducing activity and that certain substances which need oxidation arise with an accompanying reduction of the dye.

From these observations, we think that some of the endothelial cells we find in the blood are degenerating, while others are alive and performing the function of ingesting and digesting debris. The signs of degeneration are the absence of mitochondria, certain clear vacuoles, such as the one against the nucleus in Fig. 7, which are a frequent occurrence, and a ruptured cell membrane which occurs even in the supravital technique. The cell we find most frequently with a ruptured cell membrane is the smallest type; in connection with these small endothelial cells, we think that the best evidence for the dis-

crimination of them from small lymphocytes is that some have positive peroxidase granules. This will be discussed in another paper in this journal as evidence of phagocytic action. Some of the endothelial cells on our plates were clearly alive, as indicated by their motility, and by their signs of phagocytosis.

The two endothelial cells from fixed films in Figs. 11 and 12 show both the structure of the nucleus and the nature of the cytoplasm. Both of these were large cells with intact cytoplasm; in both, as in the living cell, the cytoplasm was delicate, like a thin veil. We have found that the cytoplasm is usually faintly basophilic; often it contains no granules, but again it may have few or many, reacting either with the blue or the red components of the methylene blue-azur. We interpret these granules as ingested debris, and consider that their staining reaction thus varies, both with their inherent nature and with their state of digestion. The granules of Fig. 12 are shown in blue, but they actually stained much like neutrophilic granules.

If our interpretation of these cells as desquamated endothelium is correct, then the marked variation in size would seem to indicate that they have a general origin from the endothelium of the vessels—arteries, veins, and capillaries—as well as from the specific endothelia, rather than an origin restricted to the vessels actually punctured. In one instance, in a tubercular rabbit, large sheets of endothelium were found in the circulating blood, which we think surely came from the spleen. In one sheet of desquamated endothelium 19 cells were found; in another 13. This rabbit (R 19) was killed immediately after the studies of the blood were made. The spleen was enormous, weighed 17.5 gm., and measured 10 by 2.5 by 1.5 cm. Rous and Robertson (40) give 0.68 per 1800 gm. body weight as the normal weight of the spleen in the rabbit. Supravital films of the spleen showed sheets of cells similar to those of the peripheral blood. A sheet of cells from another rabbit, shown in Fig. 10, has an entirely clear area in one of the cells, which may be one of the well known fenestrated endothelial cells from the spleen.

In 1925, Sabin, Cunningham, Doan, and Kindwall (41) reported counts of normal human blood at intervals of 15 minutes for 6 to 8 hours, in which they showed that there was a characteristic rhythm of the neutrophilic leucocytes and lymphocytes. These studies were

made with the supravital technique, but fixed films were taken at the same time. In studying the fixed films, all fragile leucocytes, that is, leucocytes with a ruptured cell wall and scattered, neutrophilic granules, and all the free nuclei, that is, the smudges that showed a definite nuclear structure, were counted as the non-motile cells. As will be seen in their table, on page 46, this gave consistently a higher figure for the non-motile cells of fixed films than of the supravital preparations, the average excess being 4 per cent.

As a continuation of this study, we made the following experiment.

Nine preparations of normal human blood were taken, in rapid succession, from the same puncture; a total white count, a supravital preparation, five pairs of cover-slips for Wright's stain, and a second supravital preparation and total white count were made. The puncture was sufficiently deep for a free flow of blood, so that there was no need of squeezing the finger throughout the experiment. The two observations for the total white count at the beginning and at the end of the experiment were 7200 and 7600 respectively. We each made one of the differential counts of the supravital preparations and got results in which the difference was within one cell per each type. We then counted 150 to 400 cells on each of the two pairs of cover-slips of the fixed films, taking the average of the two films for each interval. It was found that the fragile leucocytes, that is, those with a ruptured cellular membrane, clear nuclei, and scattered neutrophilic granules, must be counted as the non-motile, or dying leucocytes. Their number is almost always greater than the number of non-motile leucocytes of living films, but the sum of these fragile and intact leucocytes of the fixed films corresponds to the sum of the motile and non-motile leucocytes of the vital preparations. In other words, more leucocytes are fragile in the technique of pulling films than are actually in the non-motile phase of the supravital technique.

The study of the endothelial cells of fixed films is bound up with the study of smudges. It will be noted that many of the so called smudges of fixed films are compact, dense structures, staining and looking like the nuclei of the endothelial cells of Figs. 11 and 12. They show the same violet reaction in methylene blue-azur. However, when all the smudges that are clearly nuclear are counted, in such an experiment as the one recorded above, they far exceed the number of endothelial cells of the living films. To make a correlation, it is necessary to restrict the identification of endothelium strictly to cells with intact, or at least distinct and characteristic veils of cytoplasm. With these restrictions, we find endothelial cells in normal human blood up to 3 or 4 per cent and in normal rabbits up to 8 per cent.

We have, then, sought to analyze smudges with more care. In the supravital films, there are usually masses of debris, varying considerably in size and appearing more like agglutinated and degenerating platelets. Platelets, when first drawn, stain characteristically in neutral red, but this staining fades. When the debris is studied in fixed films, however, it appears much more like nuclear debris. Some smudges are clearly compact, sharply outlined, free nuclei. In Fig. 13 is shown a fragile, neutrophilic leucocyte, in which one lobe of the nucleus shows a beginning of the streaks so characteristic of some smudges. From these studies we think that smudges are largely of nuclear origin, that cytoplasm of damaged cells disintegrates more readily than the nuclei, and that the smudges are, in part, nuclei of disintegrated blood cells, in part nuclei of disintegrated endothelial cells, but they do not represent cells that were living at the moment the blood was drawn. In other words, cells damaged by the technique of making films will probably show cytoplasm as well as nuclei.

Studies have been made of the occurrence of endothelial cells in normal rabbit and human blood. From a series of twenty rabbits, 66 differential counts were made. It is our custom to puncture a vein, wipe off the first drop, and take the second if the blood is flowing freely. One hundred cells are counted, and, if no clasmatocytes are found, a second hundred are counted. In these counts, clasmatocytes, that is, endothelial cells, were present in percentages varying from 0.5 to 8 per cent in 78.7 per cent of the counts. In normal human blood, they were present up to 4 per cent in 80 per cent of the counts. In tubercular rabbits, there was a fairly constant increase in clasmatocytes up to 17 per cent. We have also found a transitory increase in them within the 1st hour after the injection of heterologous blood serum.

CONCLUSIONS.

1. There is a practically constant desquamation of endothelial cells into the circulating blood in rabbits and man.

2. These endothelial cells represent the entire range in size of endothelium from very small cells, possibly from capillaries, up to the type of the large Kupffer cell.

3. These desquamated cells can be discriminated from monocytes.

4. There are common morphological and functional characteristics among the following cells: the clasmatocytes from the specific endothelia, such as the cells from the sinuses of the liver, spleen, and bone marrow; the clasmatocytes of the diffuse connective tissues; and desquamated endothelial cells occurring in normal and pathological blood.

5. There are four strains of white cells in the normal circulating human and rabbit blood, granulocytes, lymphocytes, monocytes, and endothelial phagocytes.

BIBLIOGRAPHY.

1. Mallory, F. B., *J. Exp. Med.*, 1898, iii, 611.
2. Rowley, M. W., *New York Med. J.*, 1907, lxxxv, 674.
3. Rowley, M. W., *J. Exp. Med.*, 1908, x, 78.
4. Leede, W., *Mitt. Hamb. Staatskrankenanst.*, 1911, xii, 411.
5. Neukirch, P., *Z. klin. Med.*, 1912, lxxiv, 380.
6. Kraus, F., *Berl. klin. Woch.*, 1913, l, 1421.
7. Netoušek, M., *Folia hæmatol., 1. Teil*, 1913–14, xvii, 407.
8. Netoušek, M., *Folia hæmatol., 1. Teil.*, 1914–15, xix, 1.
9. Křiženecký, J., *Folia hæmatol., 1. Teil.*, 1916–17, xxi, 252.
10. Kaznelson, P., *Deutsch. Arch. klin. Med.*, 1919, cxxviii, 131.
11. Franco, E. E., and Ferrata, A., *Arch. sc. med.*, 1919, xlii, 109.
12. Ferrata, A., Le emopatie, Milan, 1918, i.
13. Schilling, V., *Z. klin. Med.*, 1919, lxxxviii, 377.
14. Bittorf, A., *Deutsch. Arch. klin. Med.*, 1920, cxxxiii, 64.
15. Richter, M. N., *Am. J. Med. Sc.*, 1925, clxix, 336.
16. Goldmann, E. E., Die äussere und innere Sekretion des gesunden und kranken Organismus im Lichte der "vitalen Färbung," Tübingen, 1909.
17. Goldmann, E. E., Neue Untersuchungen über die äussere und innere Sekretion des gesunden und kranken Organismus im Lichte der "vitalen Färbung," Tübingen, 1912.
18. Aschoff, L., and Kiyono, K., *Folia hæmatol., 1. Teil.*, 1913, xv, 383.
19. Aschoff, L., *Beihefte Med. Klin.*, 1926, xxii, 1.
20. Cunningham, R. S., Sabin, F. R., and Doan, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 326.
21. Sabin, F. R., Doan, C. A., and Cunningham, R. S., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 330.
22. Sabin, F. R., Doan C. A., and Cunningham, R. S., *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 125.
23. Metschnikoff, E., *Ann. Inst. Pasteur*, 1887-88, i, 321.

24. Evans, H. M., and Schulemann, W., *Folia hæmatol., 1. Teil*, 1914–15, xix, 207.
25. Evans, H. M., *Am. J. Physiol.*, 1915, xxxvii, 243.
26. Cunningham, R. S., Sabin, F. R., Sugiyama, S., and Kindwall, J. A., *Bull. Johns Hopkins Hosp.*, 1925, xxxvii, 231.
27. Evans, H. M., Bowman, F. B., and Winternitz, M. C., *J. Exp. Med.*, 1914, xix, 283.
28. Kageyama, S., *Beitr. path. Anat. u. allg. Path.*, 1925, lxxiv, 356.
29. Lewis, M. R., *Am. J. Path.*, 1925, i, 91.
30. Lewis, M. R., and Lewis, W. H., *J. Am. Med. Assn.*, 1925, lxxxiv, 798.
31. Lewis, M. R., Willis, H. S., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1925, xxxvi, 175.
32. Doan, C. A., Cunningham, R. S., and Sabin, F. R., *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 163.
33. Sugiyama, S., *Carnegie Institution of Washington, Pub. No.* 363, *Contributions to Embryology*, 1926, xviii (in press).
34. Maximow, A., *Arch. mikr. Anat.*, 1909, lxxiii, 444.
35. Sabin, F. R., *Physiol. Rev.*, 1922, ii, 38.
36. Sabin F. R., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 277.
37. Doan, C. A., *J. Exp. Med.*, 1926, xliii, 289.
38. Plantin, C. F. A., *J. Mar. Biol. Assn.*, 1923, xiii, 24.
39. Needham, J., and Needham, D. M., *Proc. Roy. Soc. London, Series B*, 1925, xcviii, 259.
40. Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1917, xxv, 651.
41. Sabin, F. R., Cunningham, R. S., Doan, C. A., and Kindwall, J. A., *Bull. Johns Hopkins Hosp.*, 1925, xxxvii, 14.

EXPLANATION OF PLATES.

PLATE 31.

FIG. 1. Desquamated endothelial cell, from the blood of a normal rabbit (R 14, Jan. 15, 1926), studied in supravital neutral red. The accompanying red cell gives the magnification. Endothelial cells in this preparation, 1 per cent.

FIG. 2. Desquamated, long endothelial cell, from the blood of a normal rabbit (R 51, Jan. 19, 1926), studied in supravital neutral red. Endothelial cells in this preparation, 1 per cent.

FIG. 3. Desquamated endothelial cell, from the blood of a rabbit (R 41, Jan. 15, 1926), 10 days after an intravenous injection of bovine tubercle bacilli; studied in supravital neutral red. Endothelial cells in this preparation, 4 per cent.

FIG. 4. Desquamated endothelial cell, of medium size, from the blood of a normal rabbit (R 15, Dec. 3, 1925), studied in supravital neutral red. Endothelial cells in this preparation, 7 per cent. The cytoplasm showed both refractive bodies and entirely unstained vacuoles; the latter we consider a sign of degeneration.

FIG. 5. Desquamated endothelial cell, of medium size, from the blood of a rabbit (R 19, Dec. 4, 1925), 18 days after intravenous injection of bovine tubercle bacilli; studied in supravital neutral red. The nucleus of the cell was almost completely divided. There were sheets of endothelium containing as many as 19 cells in the preparation from which this drawing was made.

FIG. 6. Desquamated, large endothelial cell, from the blood of a rabbit (R 16, Dec. 9, 1925), 23 days after an intravenous injection of bovine tubercle bacilli; studied in supravital neutral red. This cell had ingested a red cell, which showed the unchanged color of hemoglobin and there were three unstained masses over it. The red cell lies close to the nucleus of the endothelial cell. Endothelial cells in this preparation, 7 per cent.

PLATE 32.

FIG. 7. Desquamated endothelial cell, of medium size, from the blood of a rabbit (R 31, Nov. 30, 1925), 14 days after an intravenous injection of bovine tubercle bacilli; studied in supravital neutral red. Endothelial cells in this preparation, 1 per cent.

FIG. 8. Desquamated, large endothelial cell, from the blood of a normal rabbit (R 14, Dec. 18, 1925); studied in supravital neutral red. All of the formed bodies in the cell reacted to the dye. Endothelial cells in this preparation, 1 per cent.

FIG. 9. Desquamated, large endothelial cell, similar to a Kupffer cell, from the blood of a rabbit (R 16, Nov. 30, 1925), 14 days after an intravenous injection of bovine tubercle bacilli; studied in supravital neutral red. Endothelial cells in this preparation, 4 per cent. The outlines along the border indicate the movement of the cell while it was being drawn.

FIG. 10. Sheet of three, desquamated endothelial cells, with some platelets, from the blood of a rabbit (R 16, Dec. 19, 1925), 33 days after an intravenous injection of bovine tubercle bacilli; stained in supravital neutral red. Endothelial cells in this preparation, 13 per cent. When the drawing was started, only a few of the formed bodies were stained. The dotted outline shows the final form of the cells when the drawing was finished, indicating movement.

FIG. 11. Desquamated endothelial cell, of medium size, from the blood of a rabbit (R 19, Dec. 2, 1925), 16 days after an intravenous injection of bovine tubercle bacilli; stained with Wright's methylene blue-azur. On this day the differential count with the supravital technique showed 17 per cent of endothelial cells. The cell contained some phagocytized debris, which stained a reddish purple, though shown in gray in the reproduction; the nucleus was indented.

FIG. 12. Large, endothelial cell, from the same preparation as the cell of Fig. 11. This cell had a large, oval nucleus and a thin veil of cytoplasm.

FIG. 13. Fragile, neutrophilic leucocyte, from normal human blood, to show the nature of the degeneration of the nucleus with lines of changing chromatin, such as are characteristic of some smudges.

(Sabin and Doan: Desquamated endothelial cells in blood.)

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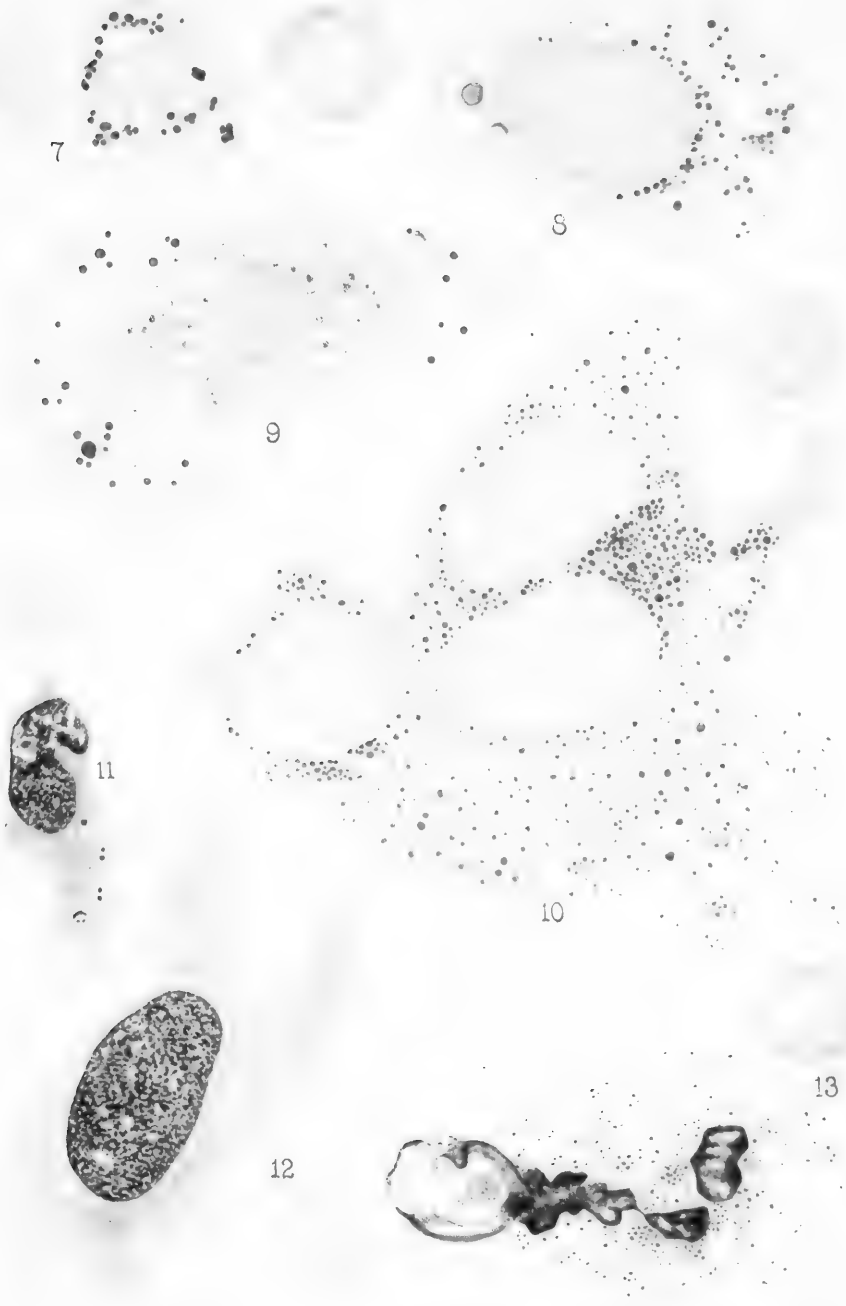

(Sabin and Doan: Desquamated endothelial cells in blood.)

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NORMAL AND PATHOLOGICAL FRAGMENTATION OF RED BLOOD CELLS; THE PHAGOCYTOSIS OF THESE FRAGMENTS BY DESQUAMATED ENDOTHELIAL CELLS OF THE BLOOD STREAM; THE CORRELATION OF THE PEROXIDASE REACTION WITH PHAGOCYTOSIS IN MONONUCLEAR CELLS.

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In 1917, Rous and Robertson (1, 2) demonstrated that there is a constant fragmentation of red cells in the circulating blood of the normal animal. They drew blood directly from the hearts of mammals into gelatin-Locke-citrate and obtained microcytes and poikilocytes by differential centrifugation. They observed that fragmentation was increased in anemia and also by such mechanical factors as pressure and heat. The process had been seen by many observers; for example, by Ehrlich (3), Arnold (4), Schwalbe (5, 6), Maximow (7), Weidenreich (8), and others, with varying interpretations, but it was Rous and Robertson who determined that it is a constant and, in their judgment, the major factor in the destruction of red cells.

We have been able to confirm the concept that there is a constant fragmentation of red cells in the circulation of the normal rabbit by the study of films of fresh blood, and have found that the fate of these fragments is to be taken up by the so called clasmatocytes or wandering endothelial phagocytes. We shall use the three terms: clasmatocyte, wandering endothelial phagocyte, and macrophage as synonymous and distinct from monocyte. Clasmatocytes with fragments of red cells are to be found in the circulating blood, in the spleen, and in the connective tissues, and we think that they can be discriminated, when studied with the supravital technique, from eosinophilic leucocytes, with which they have been confused in the literature. Thus,

clasmatocytes not only take in and destroy whole red cells, as can be demonstrated in the spleen and bone marrow of any normal rabbit, but they also dispose of the results of the fragmentation of red cells which is constantly going on in the blood stream. With any increase in fragmentation there is an increase in the number of clasmatocytes containing these fragments. If it should prove correct that the clasmatocyte is a derivative of endothelium, then it will be interesting that this strain of cells should carry the major part of the work of breaking down and conserving hemoglobin, whether by phagocytosis of whole red cells, or of their fragments, inasmuch as it is endothelium which has the power of building up hemoglobin (Doan *et al.*, 9).

To study the process of fragmentation, we have made preparations of rabbit blood on films of dilute neutral red, sealed them as rapidly as possible to limit crenation, and watched them in a warm box. Because the process is markedly increased by a rise in temperature, as was seen by Weidenreich and by Rous and Robertson, we have taken pains to note that the phenomenon can be found immediately after the preparation is made, before the film, taken at room temperature, has had time even to come to the temperature of the warm box, 37°C. Indeed, an increase in fragmentation is so constant with a rise in temperature above 37° that it serves as a check on the accuracy of the regulator in the warm box.

In watching fragmentation, we have found that a red cell puts out a long process and thereby becomes the so called poikilocyte. At first, the process does not move, then it begins to vibrate slowly, then faster and faster. This vibration marks the beginning of fragmentation. As the process vibrates, it gradually becomes thinner and thinner at some point, and then separates. If the thinned out place is at the base of the long process, the fragment becomes a rod, often looking like an irregular but yellow bacillus; if near the tip, it becomes a round or oval body. We have seen every variation in size and shape of these processes, up to an actual division of a red cell into two equal parts. Sometimes two or more processes form on a red cell at the same time. When the blood of patients with pernicious anemia is studied with reference to fragmentation, the poikilocytes show the same marked vibration of the processes as in secondary anemia, but they may not separate in supravital preparations even for 24 hours.

The blood shows many bizarre shaped fragments, nevertheless. It is thus possible that the large number of poikilocytes present in this disease may be associated with a delayed fragmentation. We interpret a poikilocyte as the first stage in the process of fragmentation, and the microcyte, in many instances at least, as a red cell reduced in size after some of its cytoplasm has broken off.

Phagocytosis of Fragments of Red Cells by Desquamated Endothelial Cells in the Blood.

The anemia which always occurs in rabbits infected with tuberculosis has been followed, and it has been found that there is an increase in fragmentation as the number of red cells and hemoglobin diminish. Fragmentation in connection with anemia was first seen by Ehrlich and studied by Rous and Robertson. In the present experiments supravital differential counts of the peripheral blood were made every other day. In the accompanying paper in this journal (10), it was shown that there is a constant desquamation of endothelial cells into the blood stream in normal rabbits and that the desquamation is markedly increased at certain stages in tuberculosis. In preparations of living blood, we have found phagocytosis of the fragments of red cells by free endothelial cells. These studies have afforded an especially good opportunity to compare the clasmatocytes with included fragments of red cells with true eosinophils. As is well known, the number of true eosinophilic leucocytes is low in rabbit blood, averaging about 2 to 3 per cent in the normal animal. In our experience, eosinophilic leucocytes disappear altogether from the blood stream in rabbits in from 24 to 72 hours after the intravenous injection of tubercle bacilli, and they almost never appear again in the blood unless the animal survives and the blood cells otherwise return to normal. After about 3 or 4 weeks, when the infected rabbits show an anemia having around 3,000,000 red cells and a hemoglobin of about 35 per cent, a few of the endothelial phagocytes in the blood appear loaded with fragments of red cells. Most of the cells are well filled with the fragments, but some have been found containing only one or two fragments. In the supravital technique it is easy to distinguish these cells from true eosinophilic leucocytes, first by the

character of the granules, and second by the different type of motility of the two cells. The eosinophilic granules of the rabbit's cells are large and oval in shape. When unstained, they are slightly greenish and highly refractive; in supravital neutral red, they react toward the orange shade shown by the dye in dilute alkaline solution. In the true eosinophilic leucocytes, there are also a few tiny red bodies in the scarlet color of the acid reaction of the dye, which we interpret as vacuoles. They are round and vary in size. The fragments of red cells, on the other hand, have exactly the color of the hemoglobin in the surrounding red cells when they have just been taken into the clasmatocyte. After they have been engulfed for a time, they begin to show a pink color in neutral red, which we interpret as the color of the fluid of digestion which the cell secretes around them. The fragments of red cells vary more in size and shape and are less refractive than eosinophilic granules. As the fragments are increasingly affected within the cell through the breaking down of the hemoglobin, they shrink and stain more and more deeply with the dye. At no stage are they like eosinophilic granules in the fresh, unstained films, or in their reaction to supravital staining.

In type of motility also the two cells are different. The true eosinophilic leucocyte has typical ameboid motion, with streaming of the granules as the cell moves rapidly across the field. The clasmatocyte, on the other hand, moves so sluggishly that one has to watch it for some minutes to see that it is changing at all.

Weidenreich (11, 12) proposed the theory that the granules in eosinophilic leucocytes are fragments of red cells. He found large numbers of eosinophilic cells in sections of hemal glands. They were within the blood spaces of the gland and in these same spaces there were large masses of fragments of red cells. He noted that the fragments outside the cells had an exactly similar reaction to Orange G and to eosin as the granules within the cells. Weidenreich discriminated between eosinophilic leucocytes and clasmatocytes with fragments of red cells by the difference in size and in the nuclei of the two cells, but he concluded that the granules were the same in both cells and that they were fragments of red cells. We think, on the contrary, that while the two types of so called granules cannot always be discriminated in fixed tissues, they can be distinguished in the living state,

both when unstained and when colored with neutral red. Concerning the presence or absence of iron in eosinophilic granules there are discrepancies in the literature, but the matter has not been considered from the standpoint of discriminating true eosinophilic leucocytes from clasmatocytes with fragments of red cells. Barker (13) found that the eosinophilic granules in leucocytes of patients with malaria and in other conditions in which the eosinophils were increased gave a reaction for masked iron. Neumann (14) has recently reported that the eosinophilic granules in the leucocytes of the horse are free from iron. Some method of discriminating these two types of cells, containing granules reacting alike to eosin, in sections of pathological material, is desirable.

In the present study the tuberculous rabbits were killed at the stage in which it was expected that a maximum grade of the disease in the lungs would be found, as indicated by the increase in modified monocytes in the peripheral blood, shown by Cunningham, Sabin, Sugiyama, and Kindwall (15). This stage coincides with a period of marked and increasing anemia. Supravital studies of scrapings of the fresh lungs, spleen, and bone marrow were made. In every instance increased numbers of clasmatocytes loaded with fragments of red cells were found in the tubercular tissue of the lungs; also they were always present in the spleen and bone marrow. Thus, it seems certain that, while marked fragmentation occurs as an evidence of increased disintegration of red blood cells in many pathological states, phagocytosis and not laking or ultimate extracellular destruction is, to say the least, a frequent fate of these fragments, as well as of the senile whole red blood cells.

One of our rabbits (R 11) showed considerable resistance to the tuberculous infection, for it was inoculated with the bacilli on November 16, 1925, and survived to March 6, 1926, when it was killed. The animal was in good condition at this time. It weighed 2020 gm. originally; the lowest weight was 1765 gm. on December 18, 1925, but the weight had returned to 1985 gm. on February 17, 1926. The red cells were originally 5,592,000, with a hemoglobin of 74 per cent (Newcomer); the greatest anemia was on December 8, 1925, with a count of 2,800,000 red cells and a hemoglobin of 44 per cent; but the red cells had returned to 6,570,000, with hemoglobin of 77 per cent the day the animal was killed. The tissues of this animal were studied for clasmatocytes with fragments of red cells; in the spleen there was the usual number of clasmatocytes with whole red cells,

but none was found with small fragments. The right lung was badly infected with large tubercular regions, and many small cavities, and in the septa of this lung many clasmatocytes were found loaded with whole red cells or with very large fragments, but none with the small fragments that could be confused with eosinophilic granules. Thus, large numbers of clasmatocytes loaded with fragments of red cells may be associated with an anemia. The left lung was normal in appearance in the gross, but showed some epithelioid cells in the septa.

Reactions of the Two Strains of Mononuclear Cells of the Blood to the Peroxidase Stain.

A method of studying blood, by which it is possible to discriminate desquamated endothelial cells from monocytes, and a condition such as tuberculosis in rabbits, in which both strains of cells are markedly increased, offer favorable material for the study of differential oxidase reactions. Concerning the oxidase reaction of mononuclear cells, there are many discrepancies in the literature.

The monocytes of human blood were described as positive to the oxidase reaction by Naegeli (16, page 144). Reschad and Schilling-Torgau (17), and Schlenner (18), using another technique, reported them negative. Reschad and Schilling-Torgau studied the oxidase reaction in monocytes in a case of monocytic leucemia in which the discrimination of the cells in fixed films was accomplished by means of the azurophilic granules, as well as by a marked development of a centrosphere around a centrosome. Schlenner studied the monocytes which are present in increased percentage in cases of malaria. In analyzing the findings of Schilling and of Schlenner, Katsunuma (19) found that the human monocytes are regularly negative with their technique, while they are positive with the original technique of Schultz. It is therefore essential to state the exact technique in every study of an oxidase reaction. Recently, McJunkin (20) has questioned the presence of any monocytes in the blood of the rabbit on the score that the so called monocytes of the rabbit are negative to the oxidase test, while the monocytes of human blood are positive. It has been shown by Katsunuma, whose recent monograph on both stabile and labile reactions in general is most comprehensive, that the monocytes of the blood of rabbits, guinea pigs, apes, dogs, rats, and mice can be regarded as negative in a general way to the stabile oxidase test, and yet they give a very weak reaction of a diffuse blue color without definite, colored granules, a reaction which can be brought out with faint illumination, in contrast with the entirely negative, stabile oxidase reaction of lymphocytes. Thus, he concludes that the oxidase substances are perhaps less ripe in the monocytes of the animals just mentioned than in human monocytes, so that they are easily overlooked. Moreover, he showed that in human blood there are always some monocyte-like cells that are negative.

Our findings agree with those of Katsunuma and provide a further analysis of the reaction in mononuclear cells.

We have used the peroxidase technique of Sato and Sekiya, as given by Sato and Yoshimatsu (21), which involves a mordant of copper sulfate, benzidine and hydrogen peroxide, and a counterstain of safranine. The method gives clear-cut results, which are constant when the blood is stained the day it is drawn; and the reagents are stabile. The counterstain of safranine gives the best differentiation we have found for identification of the type of cell involved. As is well known, the difficulty in interpreting the type of cell taking an oxidase reaction is great, because the especial characteristics by which the cells have been discriminated are not brought out. For every preparation with the peroxidase reaction we have made a differential count of consecutive drops of blood with the supravital technique and with Wright's stain, and compared the three different techniques. In the stage of tuberculosis already described as being studied, there is always a marked depression of the activity of the bone marrow, so that besides the anemia the leucocytes with fine granules are diminished, at times to as low as 12 per cent. The less massive oxidase reaction in the rabbit leucocytes makes their characteristic nuclei readily visible. Normal rabbit blood was taken for the reaction of the eosinophilic leucocytes and it was found that they show a massive blue peroxidase granulation either general or in a rim at the periphery of the cell, so that they are distinctive and markedly positive. The basophilic cells, on the contrary, which are not uncommonly as high as 10 per cent in the normal rabbit and are not reduced in tuberculosis, show bright red granules, rather than the blue granules of the other myeloid cells with the Sato and Sekiya method. At least there are cells with red granules that check in the same percentage and physical appearance with the basophils of the differential counts in the other two methods. An occasional cell in human blood shows the same reaction. The lymphocytes are, of course, uniformly negative, as in all stabile oxidase and peroxidase stains.

With the desquamated endothelial cells, there is the most interesting and varied reaction to the peroxidase stain. It is known that endothelium itself is negative. Katsunuma, however, has demonstrated that the Kupffer cells will yield a positive reaction when they

have phagocytized positive material. His experiment was as follows: he injected the positive eosinophilic granules freed from the leucocytes of the horse into the mesenteric vein of rabbits and found that the Kupffer cells, as they phagocytized this debris, became positive. In the rabbit the desquamated endothelial cells of the blood are negative or positive, according to their physiological state. As was shown by the supravital technique (10) in the accompanying paper, some of the endothelial cells in the blood do not stain in neutral red, others stain partially or completely; some are obviously degenerating, while others are motile and contain substances that can be identified as ingested debris, indicating that the cells are functioning. In the Sato and Sekiya technique, we discriminate the endothelial cells from monocytes and from lymphocytes in part by their thin, filmy cytoplasm and characteristic nuclei, and in part by their shape. We divide (10) the desquamated endothelial cells into four groups; small cells, the size of the small lymphocyte, cells of intermediate size, up to the size of the largest monocyte, very long cells, and giant cells. In the blood of tubercular rabbits there are small cells, the size of a small or intermediate lymphocyte, often 3 to 4 per cent of them, which have positive blue granules in the peroxidase films. This is confirmation that their discrimination from lymphocytes by the supravital technique is justified, since no lymphocytes have been shown with a stabile oxidase reaction.

In the studies made of the rhythm of the leucocytes from human blood, by Sabin, Cunningham, Doan, and Kindwall (22), it was found that there was a discrepancy in the counts made by the vital technique and on fixed films, which was invariably in one direction, namely, toward an increase in the cells estimated as lymphocytes, with a consequent decrease in leucocytes in fixed films, as compared with the number found by the supravital technique. The average discrepancy was about 5 per cent, but it was as high as 10 per cent in certain individual counts. The same discrepancy appears with rabbit blood. Some of the counts made by the two methods agree closely, but again there occur discrepancies in the direction of an excess of lymphocytes in the fixed films. We have now come to the conclusion that this discrepancy is due to small and medium sized clasmatocytes, which have heretofore been overlooked by us in supravital films and have

been included as lymphocytes in fixed films. We think that the free nuclei of degenerating, small endothelial cells, and perhaps of other cells, which are indistinguishable from debris in fresh films, are often included as small lymphocytes in fixed films. In the accompanying communication (10) evidence has been given to show that the majority of smudges of fixed films are of nuclear origin. To approximate accuracy in fixed films with reference to small lymphocytes, it is essential to have the original dilution of the methylene blue-azur in methyl alcohol adjusted to the particular blood to be stained, as a too intense basophilic reaction obscures the thin rim of cytoplasm in the smallest lymphocytes.

The endothelial cells of medium size, the long types, and the giant forms are negative, positive, or contain a mixture of the positive blue

TABLE I.

	No. of cells.	Total No. of endothelium.	Endothelium positive to peroxidase.	Endothelium negative to peroxidase.	Total No. of monocytes.	Monocytes positive to peroxidase.	Monocytes negative to peroxidase.
Rabbit blood (tubercular).	3200	184 5.7%	54 29%	130 71%	534 16.7%	2 0.37%	532 99.63%
Human blood (normal).	3000	64 2%	43 67.2%	21 32.8%	177 5.9%	169 95.5%	8 4.5%

granules and the red granules similar to those of the basophilic leucocytes. These cells may have one massive blue granule, two or three tiny granules, many tiny granules, or granules massively clumped or scattered throughout the cytoplasm of the cell. The clasmatocytes which are filled with fragments of red cells are negative to the peroxidase reaction in contrast to the decidedly positive reaction of the true eosinophilic leucocytes. This finding was checked in tissues where the number of these cells was known to be great from supravital studies, as well as in the peripheral blood. The percentages of the endothelial cells positive and negative to the peroxidase reaction, as compared with monocytes, are shown in Table I.

The conclusion has been reached that the desquamated endothelial cells found in the circulating blood are in part degenerating types, but in part actively functioning cells that are phagocytic in the blood

stream. Under pathological conditions these clasmatocytes have been reported as containing whole leucocytes; they probably also phagocytize fragments of leucocytes containing neutrophilic granules, which are frequently found in fresh films of blood.

It can be said of the monocytes that almost all of this type of cell in human blood are positive to the technique of Sato and Sekiya; but, as shown in the table, an occasional one is negative. In the estimation of this negative reaction, only cells of large size, with an excentric and indented nucleus, the typical transitional in the terminology of Ehrlich, were included as monocytes. That there is an occasional negative, large mononuclear cell in human blood agrees with the findings of Katsunuma. Moreover, with the positive monocytes of human blood, there is the greatest possible variation in the number of the blue granules, from a reaction of numerous fine granules, scattered throughout the cytoplasm, down to one positive granule. With the monocytes of the blood of rabbits the reverse condition is true, because the vast majority of their monocytes lack the granules and only an occasional cell is positive. Here also the greatest care must be taken in the study to exclude two other types of cells, namely, large lymphocytes and clasmatocytes of appropriate size. Only a cell quite clearly of the type of the transitional cell of Ehrlich was considered, a large cell with dense cytoplasm and with an excentric and indented nucleus.

We therefore conclude that most of the monocytes of human blood have positive peroxidase granules with great variation in the number of these granules, but that a few monocytes lack them entirely; while, in contrast, most of the monocytes of rabbit blood lack the positive blue granules, but a few contain them. Thus, the difference in monocytes in various species seems to be more one of degree than of kind, and therefore not a basis for a classification, but rather an indication of a difference in functional activity. The epithelioid cells of the tuberculous lesions in the rabbit have been found negative, as in the case of the monocytes from which we believe they are derived, except in rare instances, in which a leucocyte containing positive granules had clearly been phagocytized. The monocytes, epithelioid cells, and giant cells from human tubercular lymph glands have[1] been found

[1] Through the courtesy and cooperation of Dr. John Hanford, of the Surgical Staff of the Presbyterian Hospital, we have had access to fresh human tubercular lymph glands (cervical) removed at operation.

to be positive to the peroxidase reaction, having fine blue granules comparable to the reaction in the 15 per cent of positive monocytes found in the peripheral blood of the same patient the same day. Thus, the essential identity of the monocytes of rabbit and human peripheral blood and their respective relationship to the specific cellular reaction in tuberculosis would seem to be entirely compatible with the peroxidase reaction occurring in these cells. Clasmatocytes filled with fragments of red blood cells and negative to the peroxidase reaction were conspicuously present in these human lymph gland preparations.

CONCLUSIONS.

1. There is constantly some breaking down of red cells in the circulation by fragmentation.

2. The fragments of red cells, as well as whole red cells, are phagocytized and destroyed by clasmatocytes or endothelial phagocytes.

3. When there is an increase in fragmentation in abnormal or pathological states, desquamated endothelial cells of the blood stream, as well as the clasmatocytes of the tissues, increase proportionately and take in these fragments. These cells are to be distinguished from eosinophilic leucocytes by the nature of their granules, by the type of motility of the cells, and by a negative peroxidase test.

4. The desquamated endothelial cells, clasmatocytes, in the circulating blood are positive to the peroxidase test only when they have taken in positive material.

5. The monocytes show marked variations of the oxidase reaction in different species and to different techniques. With the Sato and Sekiya technique most monocytes of human blood are positive, while most of them in rabbit blood are negative, but both positive and negative reactions are found in both human and rabbit blood.

BIBLIOGRAPHY.

1. Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1917, xxv, 651; *Studies from The Rockefeller Institute for Medical Research*, 1917, xxvii, 163.
2. Robertson, O. H., and Rous, P., *J. Exp. Med.*, 1917, xxv, 665; *Studies from The Rockefeller Institute for Medical Research*, 1917, xxvii, 177.
3. Ehrlich, P., Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes, Berlin, 1891, pt. 1, 99.

4. Arnold, J., *Virchows Arch. path. Anat.*, 1899, clv, 165.
5. Schwalbe, E., *Ergebn. allg. Path. u. path. Anat.*, 1902, viii, 150.
6. Schwalbe, E., *Beitr. path. Anat. u. allg. Path.*, 1905, suppl. 7, 52 (Festschrift für Dr. Julius Arnold).
7. Maximow, A., *Arch. Anat. u. Physiol., Anat. Abt.*, 1899, 33.
8. Weidenreich, F., *Anat. Anz.*, 1903–04, xxiv, 186.
9. Doan, C. A., Cunningham, R. S., and Sabin, F. R., *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 163.
10. Sabin, F. R., and Doan, C. A., *J. Exp. Med.*, 1926, xliii, 823.
11. Weidenreich, F., *Anat. Anz.*, 1901, xx, 188.
12. Weidenreich, F., *Anat. Anz.*, 1901–02, xx, 193.
13. Barker, L. F., *Bull. Johns Hopkins Hosp.*, 1894, v, 93.
14. Neumann, A., *Z. wissensch. Biol., Abt. B*, 1925–26, iii, 46.
15. Cunningham, R. S., Sabin, F. R., Sugiyama, S., and Kindwall, J. A., *Bull. Johns Hopkins Hosp.*, 1925, xxxvii, 231.
16. Naegeli, O., Blutkrankheiten und Blutdiagnostik, Berlin, 4th edition, 1923.
17. Reschad, H., and Schilling-Torgau, V., *Münch. med. Woch.*, 1913, lx, 1981.
18. Schlenner, F., *Deutsch. med. Woch.*, 1921, xlvii, 6.
19. Katsunuma, S., Intrazelluläre Oxydation und Indophenolblau-synthese, Jena, 1924.
20. McJunkin, F. A., *Arch. Int. Med.*, 1925, xxxvi, 799.
21. Sato, A., and Yoshimatsu, S., *Am. J. Dis. Child.*, 1925, xxix, 301.
22. Sabin, F. R., Cunningham, R. S., Doan, C. A., and Kindwall, J. A., *Bull. Johns Hopkins Hosp.*, 1925, xxxvii, 14.

ETIOLOGY OF OROYA FEVER.

I. Cultivation of Bartonella bacilliformis.

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Plates 33 to 35.

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The microbic incitant of Peruvian Oroya fever has been recognized by previous investigations.

Barton,[1] especially, detected the bacilliform "X" bodies which occur constantly in the red corpuscles of cases of the infection and distinguished them accurately from the non-specific basophilic rods and granules present in the red corpuscles in other diseases. Strong, Tyzzer, Sellards, Brues, and Gastiaburu,[2] who also detected the bacilliform bodies, regarded them as motile microorganisms probably of protozoan nature which invade both red corpuscles and vascular endothelium; and they proposed for them the name of *Bartonella bacilliformis*. *Bartonella bacilliformis* would seem to be the specific cause of Oroya fever, as malaria plasmodia are for malaria, despite the fact that the organism has been found in certain cases of verruga peruviana. While this association of two clinically dissimilar diseases has proved disturbing, Peruvian investigators had connected Oroya fever with verruga long before the bacilliform organism was discovered. According to Odriozola,[3] verruga may be present when Oroya suddenly supervenes, or both maladies may arise simultaneously, or verruga may follow Oroya. These facts have led many Peruvian investigators to regard verruga and Oroya as different manifestations of the same disease, a conclusion supported by the heroic deed of Carrion, who, believing in a common etiology for verruga and Oroya, submitted himself to inoculation with verruga, the result being the development of a clinical case of fever, accompanied by the usual severe anemia, to which he succumbed.

We are in a relatively favorable situation today for deciding on the relationship of Oroya fever and verruga. The improvements in bac-

[1] Barton, A. L., *Crón. méd.*, 1909, xxvi, 7.

[2] Strong, R. P., Tyzzer, E. E., Sellards, A. W., Brues, C. T., and Gastiaburu, J. C., Report of first expedition to South America, 1913, Harvard School of Tropical Medicine, Cambridge, 1915.

[3] Odriozola, E., La maladie de Carrion, Paris, 1896.

teriologic technique of the last few years have led to the cultivation of a number of microorganisms previously resisting culture. It was regarded as desirable that these methods be applied to Oroya fever. There can no longer be doubt that it and verruga can exist together, either one preceding in appearance the other. Moreover, it has been thoroughly demonstrated[4,5] that the monkey may be successfully inoculated intradermally from cases of verruga. The possibility exists, therefore, of testing by inoculation the power of cultures derived from cases of Oroya fever to set up the lesions of verruga in animals.

We have applied the method originally devised for the cultivation of leptospiras[6] and leishmanias[7] to blood derived from a case of Oroya fever (Santa Anna 15) in the service of Dr. Olaechea, of the Dos de Mayo Hospital, Lima. Through the generous permission of Dr. Olaechea, a blood sample was withdrawn on September 4, 1925; the patient died 2 days later from the severe progressive anemia usual in the disease.

Several culture tubes were inoculated with the fresh citrated blood at Lima, and these tubes, together with the remainder of the citrated blood, were brought back to New York, the date of departure from Lima being September 9, and of arrival in New York September 21. The blood was kept at refrigerator temperature during the entire period after its collection from the patient.

Bacterial contaminations occurred in the original culture tubes during the journey, but renewed attempts at cultivation with the citrated blood yielded a pure culture of *Bartonella bacilliformis*, which has been employed in the studies to be reported here. The media listed in Table I were used, with the results noted.

Each tube was inoculated with 0.05 cc. of the citrated blood, placed at 37°C. for 4 days, and then removed to room temperature for 4 days or more. Only a trace

[4] Jadassohn, G., and Seiffert G., *Z. Hyg. u. Infectionskrankh.*, 1910, lxvi, 247.

[5] Mayer, M., Rocha Lima, H., and Werner, H., *Münch. med. Woch.*, 1913, lx, 739.

[6] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13; *J. Trop. Med.*, 1925, xxviii, 185.

[7] Noguchi, H., *Proc. Internat. Conf. Health Prob. Trop. America*, Kingston, Jamaica, British West Indies, July 22 to August 1, 1924, published in Boston, 1924, p. 455.

of opalescence occurred on the surface of the tubes of leptospira medium, and they were at first set aside as negative. On reexamination a few days later, however, numerous minute organisms were recognized. Examination by dark-field microscope, as well as staining with Giemsa's and Gram's solutions, were utilized for the detection of the organism.

TABLE I.

1. Leptospira medium. 0.9 per cent NaCl..... 800 parts. Fresh rabbit serum..... 100 " 2 per cent nutrient agar (pH 7.4)..... 100 " Rabbit hemoglobin (made by laking 1 part of defibrinated blood with 3 parts of distilled water)..... 10–20 "	Growth on surface layer.
2. Same, containing fresh tissue.	" " " "
3. Leptospira medium containing glucose, maltose, inulin.	" " " "
4. Same, plus fresh tissue.	" " " "
5. Horse blood agar slant. Defibrinated horse blood added to melted 2 per cent nutrient agar (pH 7.4) to give concentration of 20 per cent.	" " " "
6. Same, containing glucose, maltose, inulin.	" " " "
7. Plain agar slant.	No growth.
8. Plain broth.	" "

Bartonella bacilliformis in Culture.

Growth and Morphology.—Bartonella bacilliformis grows on solid or semisolid media containing blood of the rabbit, horse, or man; in a fluid medium it never multiplies sufficiently to be readily recognized. No growth can be obtained on ordinary slants or broth. Growth on blood slants is difficult to recognize, since the colonies are extremely minute and transparent, hardly visible to the naked eye (Fig. 2). Cultures on the semisolid leptospira medium, when the number of organisms introduced is large, are more readily detected by

a grayish haziness or punctiform colonies in the top layer. After 1 to 2 weeks at 28°C. the growth extends as deep as about 1 cm. below the surface (Fig. 1, *a*), and resembles somewhat in appearance the growth of leptospiras. When, on the other hand, the number of organisms inoculated is small, several very minute, discrete, grayish white granules first appear, and as time goes on each colony becomes gradually surrounded by a hazy zone (Fig. 1, *b*). This phenomenon is never seen in cultures of any of the leptospiras. The organisms grow at 37°C. as well as at 25–28°C., but at the lower temperatures they continue to grow and survive for a much longer time. In this respect *Bartonella bacilliformis* behaves much as do various flagellates and leptospiras, which can be most conveniently cultivated and maintained at the lower temperatures. No growth takes place under anaerobic cultural conditions.

The hydrogen ion concentration in which growth occurs lies between pH 6.8 and 8.4, but the organisms grow best at pH 7.8. No morphological variations due to varying the hydrogen ion concentration have thus far been detected. None of the common carbohydrates (dextrose, saccharose, galactose, maltose, levulose, xylose, lactose, mannose, mannitol, dulcitol, arabinose, raffinose, rhamnose, dextrin, inulin, salicin, and amygdalin) are fermented. Erythrocytes contained in culture medium are not hemolyzed by the growth of the organism.

When grown on blood slants at 25°C. the organisms are motile for many days and are rather uniformly rod- or spindle-shaped. Aggregates of a few to several hundred organisms are usually seen, both in fresh preparations by dark-field illumination, and in stained ones. By dark-field illumination, most of the young forms are seen to be actively motile, those aggregated into masses moving along slowly, the free individuals dashing swiftly across the field. In the leptospira medium are found small coccobacillary forms fairly well separated from one another when embedded in the medium. Many aggregates of organisms are also observed (Fig. 3). They are less motile in this medium and lose their motility usually within 1 to 2 weeks at 28°C. Pleomorphism prevails, and huge abnormal spirochete-like flagella are visible under the dark-field microscope in such cultures (Fig. 4, *a*). The normal flagella of active organisms are invisible except when stained by special methods (Fig. 4, *b*).

The organisms appear larger in fresh preparations viewed by dark-field illumination than after staining, and young forms show a definite double contour (Figs. 3, *a* and *b*). The rods are not always straight; sometimes they show a slight bend, and they are often massed together in aggregates of from two or three to a hundred. In older cultures the double contour effect is lost, the organisms appearing as decidedly thinner rods, agglomerated into masses in innumerable numbers (Fig. 3, *c*). They are now no longer motile.

The microorganism is Gram-negative and stains reddish violet with Giemsa's solution (Figs. 5 and 6). After Giemsa's staining, however, the sharp outlines are lost, and the organisms appear ill defined as if through degeneration. Minute punctiform, spindle-shaped, or oval elements, some in chains or in irregular entanglements, may be found. There are also rather large and irregular forms, densely packed in masses and taking a deeper stain. All the forms seen within the red corpuscles of Oroya patients (Fig. 7, *a*, *b*, and *c*) are also to be found in cultures. The stained specimens of cultures vary in length from 0.3 to 2.5μ—exceptionally to 3μ—and in width from less than 0.2 to 0.5μ. Chains of several individuals are also seen. Coarse oval forms measure about 0.7 by 1.2μ.

Inoculation Effects.

Inoculation of the cultures into various animals (ringtail, ***rhesus***, Java, and green monkeys, dogs, rabbits, mice, rats, and guinea pigs) were undertaken, but the results thus far have been definite only in the ***rhesus*** monkeys. The following protocols and charts represent the findings in these animals.

Macacus rhesus 1 (Chart 1; Fig. 9). November 16, 1925, received intravenously 2 cc. of cultures from leptospira medium (9 days old) and sugar blood slant (4 days old). At the same time the eyebrows were intradermally inoculated with the same material at two sites on each side. The animal remained apparently well until 8 days later, when it seemed less active. The temperature never rose above 104.8°F. at any time during the 55 days of observaton, the type of fever being irregularly remittent and very mild. Areas of induration became evident at the sites of intradermal injection after 10 to 14 days, and some of these developed into reddish nodules approximately 6 by 4 mm. in diameter.

Blood was withdrawn 13, 22, 33, and 43 days, after the inoculation. Cultures made with the blood yielded, on each occasion, a pure growth of *Bartonella bacilli-*

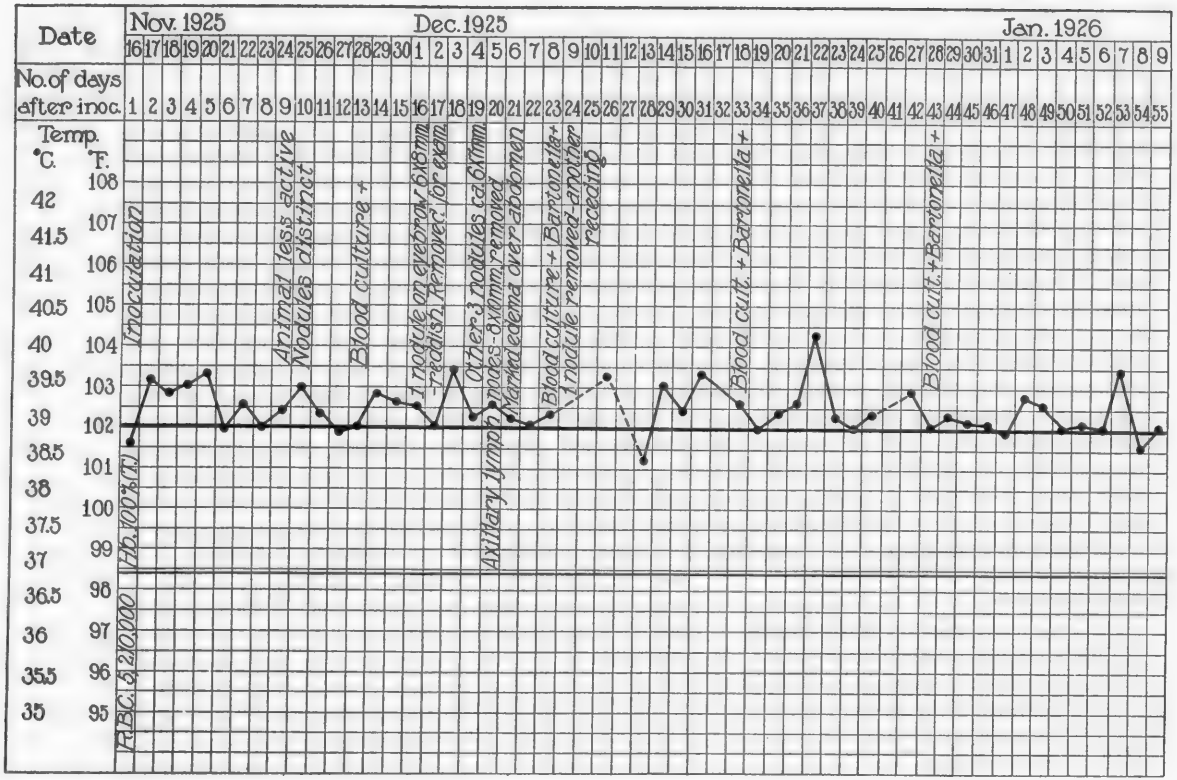

Date
Nov. 1925
Dec. 1925
Jan. 1926
No. of days after inoc.
Temp. °C. °F.
Inoculated ian
Animal less active
Nodules distinct
Blood culture +
1 nodule on eyebrow, 6x5mm - reddish. Removed for exam.
Others 3 nodules ca 6x4mm
Axillary lymph nodes - 8x4mm, removed
Marked edema over abdomen
Blood culture + Bartonella +
1 nodule removed - another receding
Blood cult. + Bartonella +
Blood cult. + Bartonella +
Wt. 10 3/4 (4)
RBC. 3 210 000

CHART 1. *Macacus rhesus* 1.

formis. A small number of intracorpuscular forms of the organism were demonstrated in blood smears (Fig. 8, *a*). One of the nodules, which was excised[8] 16 days after inoculation, showed typical infiltration of mononuclear phagocytes but was negative for *Bartonella bacilliformis*. The blood withdrawn 23 days after inoculation was transferred to three normal *Macacus rhesus* monkeys.

Macacus rhesus 2 (Chart 2). December 8, 1925, received intravenously 6 cc. of a mixture containing cultures of the 1st, 2nd, and 3rd generations derived from the blood of *Macacus rhesus* 1, as well as citrated blood withdrawn from the same animal on two occasions (the day of inoculation and 11 days previously). At the same time the left eyebrow was injected intradermally with the mixture and the right eyebrow with the emulsion of the nodule freshly excised from *Macacus rhesus* 1. The temperature rose to 104.3°F. on the 5th day and remained at about that point for 3 days. The fever in this animal fluctuated irregularly, the highest point reached being 105°F. on the 23rd day. There were several times during the 50 days of observation when the temperature fell to subnormal. The culture made with blood withdrawn on the 1st day of fever yielded a pure growth of *Bartonella bacilliformis*, as did also those made with blood withdrawn 9, 11, 21, 28, and 36 days after inoculation; and typical forms of the organism were found in a few of the red corpuscles (Fig. 8, *b*). At the sites of inoculation on the left eyebrow definite nodules had developed after about 28 days and these continued to increase in size during the following weeks.

Macacus rhesus 3 (Chart 3; Fig. 16). Inoculated at the same time and in the same way as *Macacus rhesus* 2. The course of disease was similar to that in the foregoing animal, except that the febrile reaction was much more violent during the first 5 weeks. It was similarly irregular, however, and remittent. The two sites of intradermic inoculation with culture on the left eyebrow began to show induration within about 10 to 14 days. Only one of the two inoculations on the right eyebrow (inoculated with the emulsion of a nodule of *Macacus rhesus* 1) gave rise to a nodule, and induration became evident only after 28 days, the evolution of the lesion being much slower than that of nodules produced with culture material. All three nodules were still increasing in size when examined 45 days after inoculation. One became pedunculated and cherry-red after 2 months and subsequently softened at the center. A yellowish fluid began to exude from it 68 days after the inoculation.

Macacus rhesus 4 (Chart 4; Fig. 17). The date of injection and the material injected were the same as in the foregoing two animals. The reaction was somewhat more severe than in the others, the temperature being 104°F. on the day following the injection and reaching 105.4° on the 7th day. On the 16th day the fever was interrupted, the temperature falling to slightly subnormal for 1 day. 13 days of fever followed, and the temperature was 103.4° on the 23rd day, when the animal died while under anesthesia during removal of one of the nodules. No sign of tuberculosis could be found at autopsy. The spleen was enlarged. The

[8] All operations were performed under ether aneshesia.

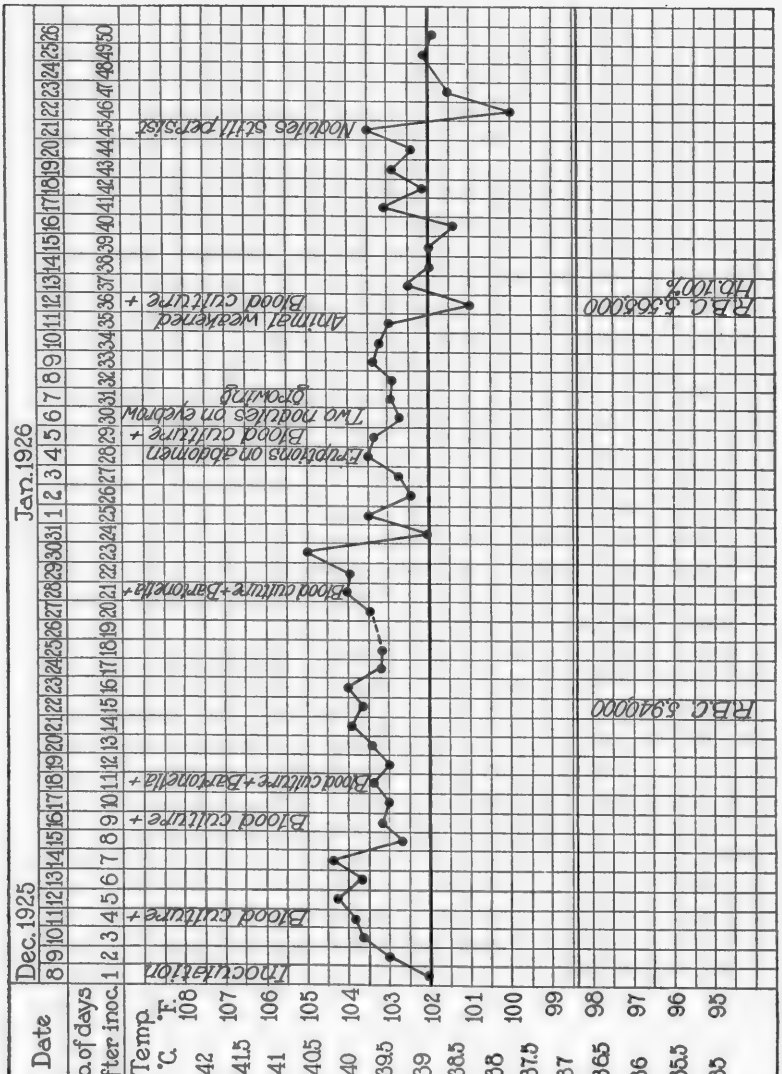


CHART 2. *Macacus rhesus* 2.

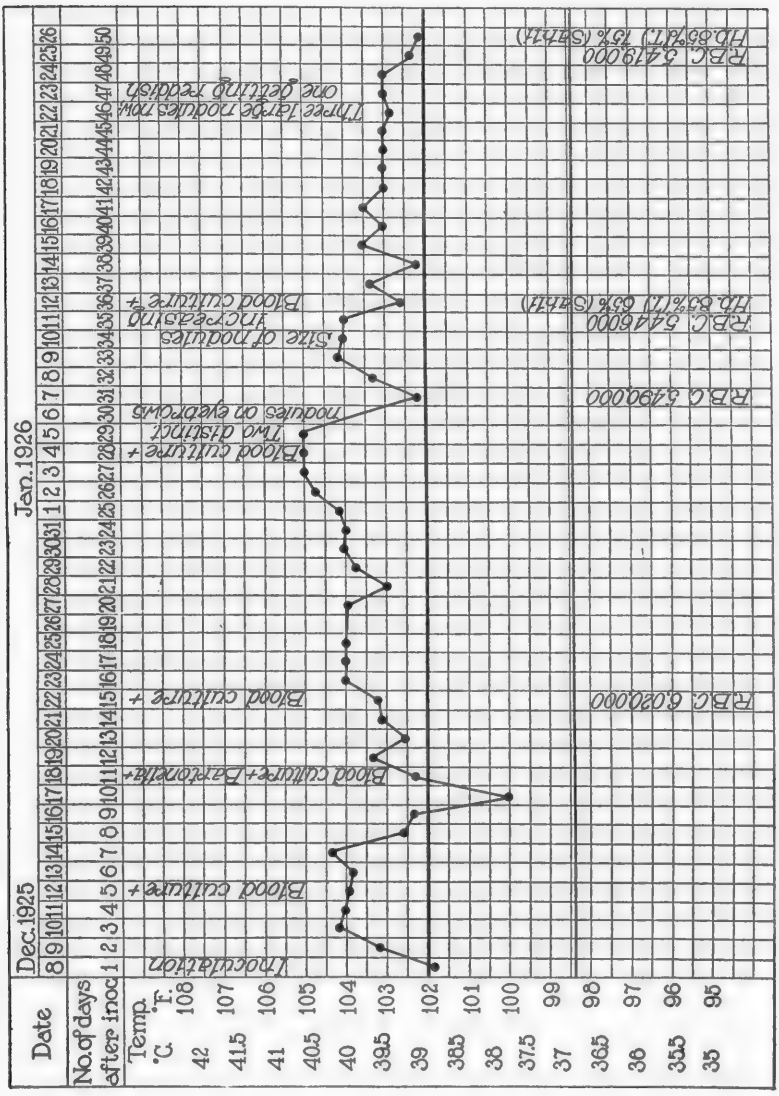


CHART 3. *Macacus rhesus* 3.

lymph glands were generally enlarged, but there were no lesions in the lungs. Pure cultures of *Bartonella bacilliformis* were obtained from blood withdrawn 5, 7, 11, 14, and 21 days after the inoculation, and from blood, spleen, and lymph nodes taken at autopsy. *Bartonella bacilliformis* was also found in small number in the red corpuscles (Fig. 8, *c*). The nodules excised 13 and 23 days after inoculation showed edema, and endothelial and capillary proliferation (Figs. 9 and

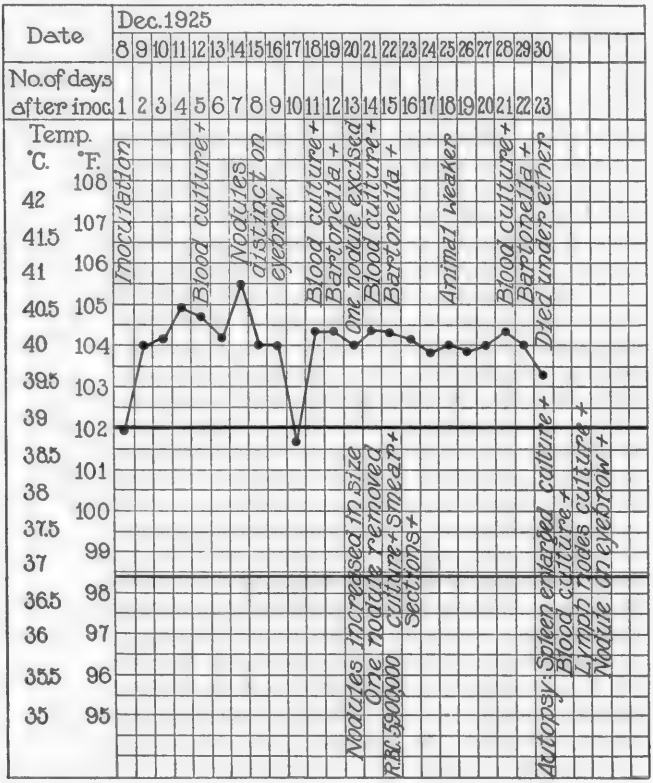


CHART 4. *Macacus rhesus* 4.

10), the microorganisms being demonstrated in cultures, smears, and sections (Figs. 13 to 15), as well as by dark-field examination of the fresh emulsion (Fig. 11). No tubercle bacilli were found in any of the tissues examined.

Further observations on the surviving monkeys, as well as the results

of other animal inoculations, will be reported later. The findings here described suffice to show that a culture of *Bartonella bacilliformis* obtained from the blood of an Oroya fever patient induces in young *rhesus* monkeys, when inoculated intravenously, a peculiar, irregularly remittent, and sometimes severe type of fever, and when inoculated intradermally into the eyebrow, gives rise to a nodule rich in cellular elements and capillary formation. Control inoculations have been made with the culture medium employed for growing the organism, and with cultures killed by heating at 65°C. for 15 minutes. Neither of these materials led to any of the changes induced by the living organisms.

Bartonella bacilliformis persists in the blood of the infected animals for many weeks, as shown by blood cultures and smears. Intracellular forms of the organism are readily found in the proliferated endothelial cells in the nodules, from which pure cultures are also to be obtained.

The infection in monkeys, unlike Oroya fever in man, has not thus far given rise to anemia, and the number of infected red corpuscles is very small, prolonged search being required to detect one. The lymph glands and spleen, however, become enlarged, and pure cultures of *Bartonella bacilliformis* have been isolated from them.

Inoculation into *rhesus* monkeys of the citrated human blood from which our cultures were derived, after it had been kept 18 days in the ice box, induced no apparent symptoms, but since the blood of these animals was not tested culturally the success or failure of the inoculations cannot be definitely stated. It is possible that overwhelming numbers of young and active organisms, such as are present in cultures, are necessary to break down the normal resistance of the monkeys, which is considerable. The relatively high resistance of such animals, as compared with man, may explain also the fact that pronounced anemia does not occur in the experimental infection. Further study upon these points is necessary.

Filtration experiments with cultures have been negative.

SUMMARY.

A pure culture of a microorganism resembling in morphology and pathogenic action *Bartonella bacilliformis* has been obtained from blood

taken during life from a case of Oroya fever which ended fatally. The blood taken at Lima into citrate solution and transported to New York at refrigerator temperature yielded positive cultures 28 days after its withdrawal from the patient.

The strain of *Bartonella bacilliformis* thus isolated grows well on the semisolid leptospira medium, and also on slant agar containing animal blood. The initial growth is not readily recognizable to the naked eye, but the presence of the organisms can be determined by means of the dark-field microscope and by Giemsa and Gram staining methods. No growth has been obtained on the more ordinary culture media. The organism is an obligate aerobe, is Gram-negative, and under certain cultural conditions motile. All the forms which have been described as occurring in human red corpuscles may be found in the cultures, and in addition many granular and coarsely irregular forms have been met with.

The inoculation of cultures of *Bartonella bacilliformis* into *Macacus rhesus* produces infection and gives rise to effects which differ with the mode of inoculation. The intravenous injection of the culture into young macaques induces a prolonged irregularly remittent fever. The organism can be cultivated from the blood over a long period, and it has been detected within the red corpuscles of the monkeys, reproducing the precise appearances observed in human cases of Oroya fever.

The intradermal injection of the culture into the eyebrow of young macaques gives rise to nodular formations rich in new blood vessels and showing the bacilliform organism within the endothelial cells. From the experimentally induced nodules cultures of the organism are readily recovered.

EXPLANATION OF PLATES.

PLATE 33.

FIG. 1. Appearance of growth of *Bartonella bacilliformis* on leptospira medium inoculated (*a*) with 0.01 cc. of the citrated blood of *Macacus rhesus* 4 and (*b*) with 0.00001 cc. Fig. 1, *b* shows several discrete hazy colonies. Cultivation at 28°C. for 14 days.

FIG. 2. Appearance of surface colonies of *Bartonella bacilliformis* on sugar blood agar plate, cultivated at 28°C. for 4 days. The colonies were so minute and so

nearly transparent that they had to be photographed by passing the light obliquely over the plate. Hence they appear as white spots, owing to the reflection of light by them. × 4½.

FIG. 3. Dark-field views of *Bartonella bacilliformis* in cultures grown at 28°C. (*a*) on agar blood slant for 5 days, (*b*) on leptospira medium for 7 days, and (*c*) on agar blood slant for 5 days. Organisms with definite double contour are shown in (*a*) and (*b*), while (*c*) illustrates the tendency of the organisms to agglomerate into huge masses, in which it is difficult to distinguish individuals. × 1000.

FIG. 4, *a*. Two detached gigantic involuted flagella of *Bartonella bacilliformis* in a culture grown on leptospira medium for 10 days at 28°C. × 1000.

FIG. 4, *b*. Young organisms with flagella (6 days on blood slant at 28°C.). Flagella stain. × 1000.

FIG. 5. Culture forms of *Bartonella bacilliformis* grown on blood agar slant at 28°C. for 4 days. Giemsa's stain. × 1000.

FIG. 6. Cultures forms of *Bartonella bacilliformis* grown on leptospira medium at 28°C. for 5 days. Giemsa's stain. × 1000.

PLATE 34.

FIG. 7, *a* to *c*. *Bartonella bacilliformis* in human red blood corpuscles. The smear was made from the blood of the patient from whom our strain was isolated. Several extracorpuscular forms are also shown. Giemsa's stain. × 1000.

FIG. 8. *Bartonella bacilliformis* in red blood corpuscles of (*a*) *Macacus rhesus* 1, (*b*) *Macacus rhesus* 2, and (*c*) *Macacus rhesus* 4, all of which were inoculated with culture. Giemsa's stain. × 1000.

FIG. 9. *Macacus rhesus* 1, showing three well developed nodules on the left eyebrow 16 days after intradermic inoculation of culture of *Bartonella bacilliformis*.

FIG. 10. Section showing the structure of one of the nodules produced by intradermic injection of culture of *Bartonella bacilliformis* isolated from the blood of *Macacus rhesus* 1. The nodule was removed 23 days after the inoculation. Regaud's fixation, Giemsa's stain. × 182.

FIG. 11. *Bartonella bacilliformis* in an emulsion of one of the nodules of *Macacus rhesus* 4. Dark-field view. × 1000.

FIG. 12. *Bartonella bacilliformis* in a smear of the same emulsion. Giemsa's stain. × 1000.

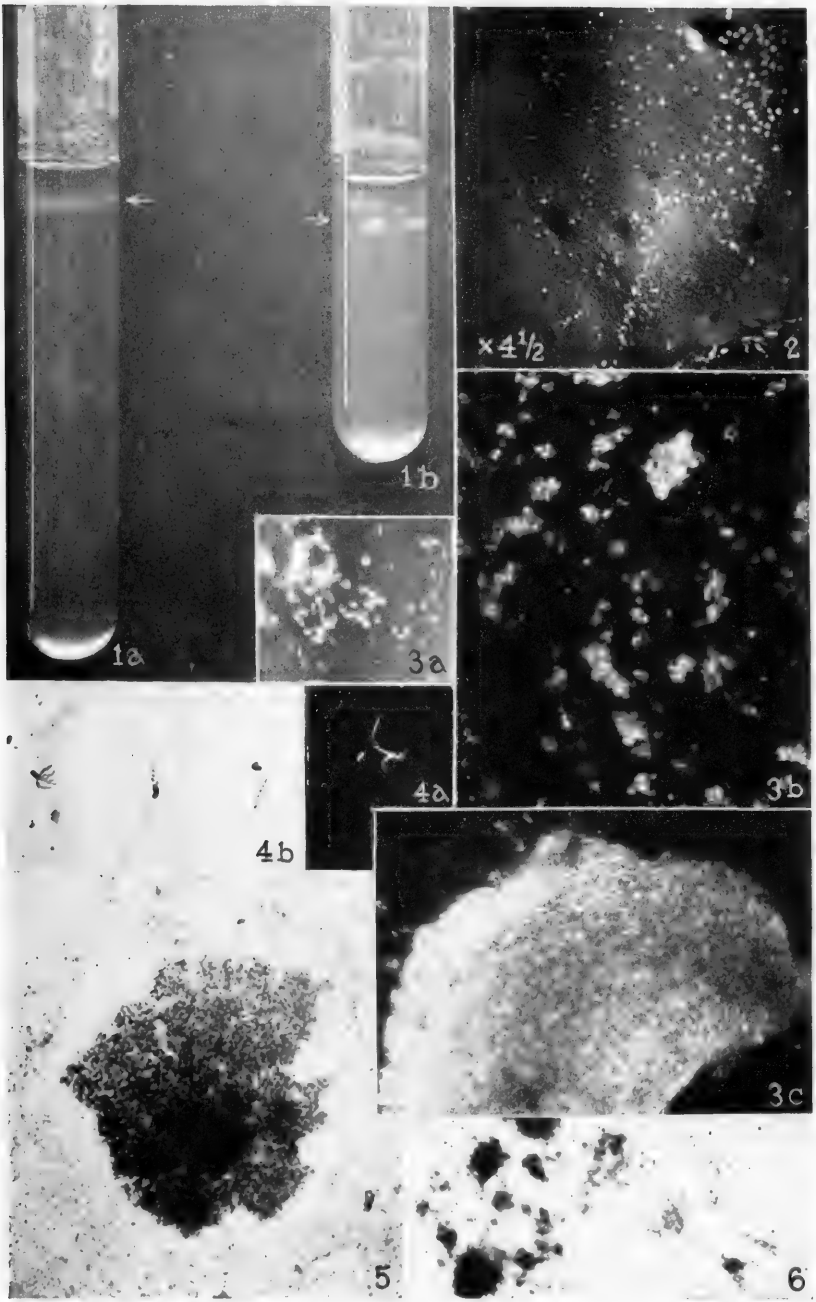
FIGS. 13 to 15. *Bartonella bacilliformis* in the angioblasts and endothelial cells of newly forming capillaries. Sections fixed in Regaud's and stained with Geimsa's solution. × 1000.

PLATE 35.

FIG. 16. *Macacus rhesus* 3. The nodule on the right eyebrow developed after the intradermic inoculation of an emulsion of a nodule excised after 16 days from *Macacus rhesus* 1. The picture shows the natural size and color of the nodule 45

days after inoculation, when it was excised for histological and cultural study and for further passage. The two nodules on the left eyebrow resulted from the injection of cultures obtained from the blood of *Macacus rhesus* 1. The picture shows their size and color 2 months after inoculation. The pedunculated nodule shows a tiny opening at the apex; it subsequently became softened at the center, and 68 days after inoculation a yellowish fluid began to exude. It was excised the following day (February 15, 1926) for sectioning, cultural studies, and transfer. Life size.

FIG. 17. *Macacus rhesus* 4. The appearance of the nodules 23 days after intradermic inoculation of the same culture as was injected into *Macacus rhesus* 3. The nodule near the median line stands out prominently and shows two round indurations. The epidermis is expanded and shiny. There was no softening. The nodule at the outer end of the left eyebrow was less circumscribed, but it was more deep seated, extending into the surrounding subcutaneous tissues. It was of firm consistency, like the others. The sites of inoculation, on the right eyebrow, of the emulsion of nodule tissue from *Macacus rhesus* 1 show scarcely a trace of induration. Life size.



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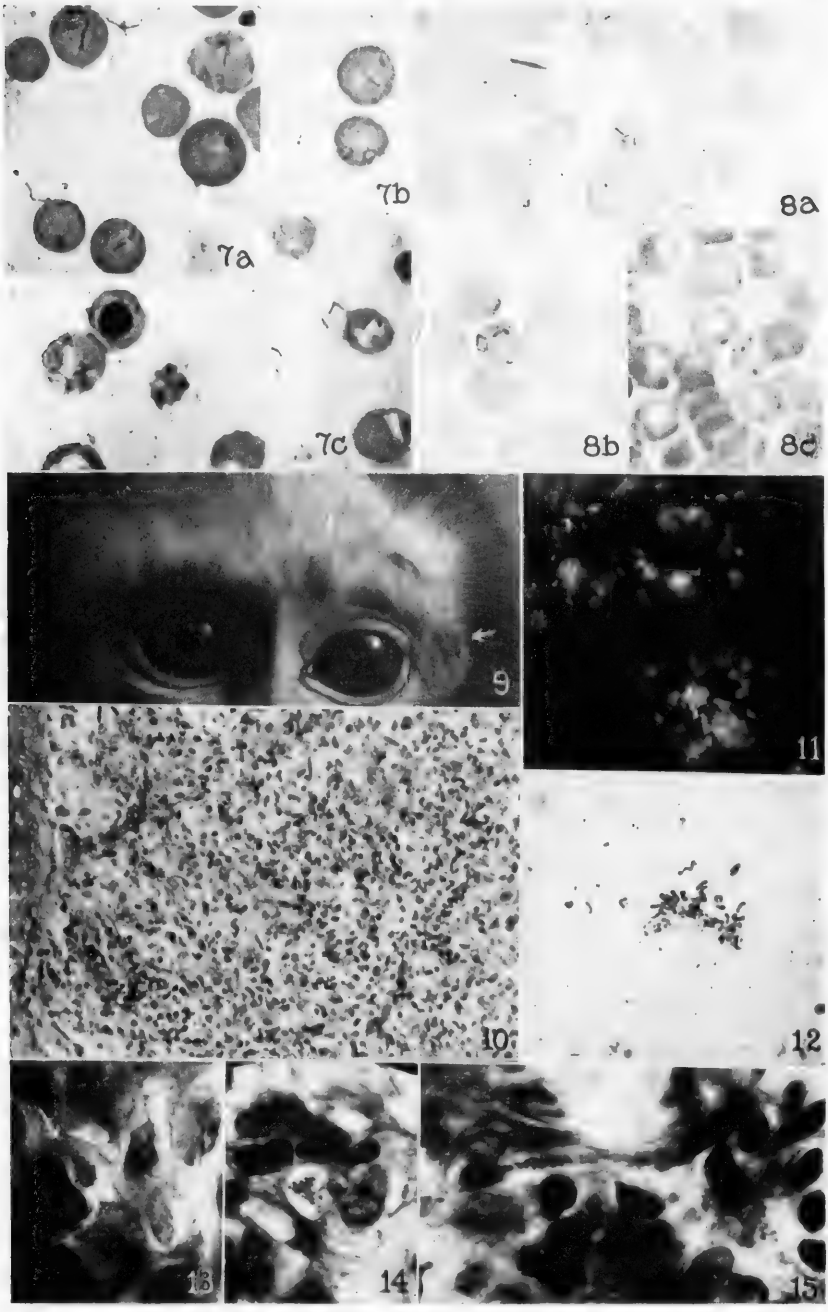

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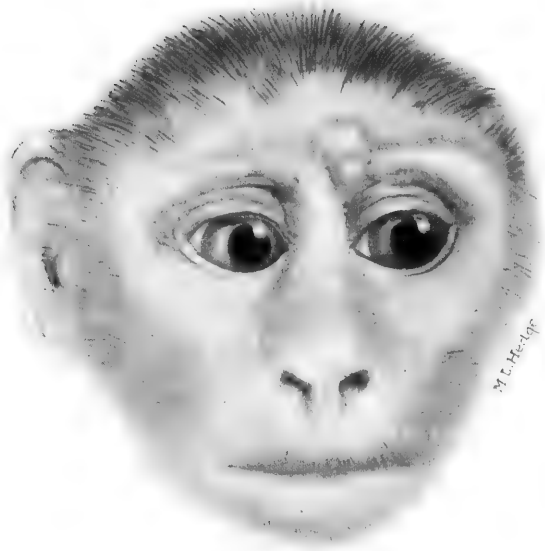

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